A NEUTRAL PROTEASE OF THE NEUTROPHIL SURFACE: ROLE IN THE PROTEOLYSIS OF C-REACTIVE PROTEIN AND FIBRINOGEN

by

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Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY in the Faculty of Medicine

UNIVERSITY OF CAPE TOWN

March 1995

To my parents and Ian

ACKNOWLEDGEMENTS

During the course of this thesis I have recieved assistance and support from people too numerous to be mentioned here and would like to thank all those who have made the journey easier. In particular, I would like to mention:

- Associate Professor Enid Shephard, my supervisor, for expert guidance, support and encouragement beyond the call of duty. Her dedication and devotion to scientific research has been a constant source of inspiration.

- Professor Ralph Kirsch for his valuble input, computer expertise, stimulating discussion and his unique ability to 'see through the clouds' and instil confidence.

- Dr Susan Adams for her friendship, encouragement and thorough proof reading of this thesis.

- Lesley Frith and Val Hancock for their continuous supply of purified fibrinogen.

- Associate Professor Wolf Brandt, in the Department of Biochemistry, UCT, for performing the amino acid analysis.

- Phillipa at Medical Graphics, UCT, for expertly handling the artwork and photography.

- Mick Wells for his unique technical ability, calm manner during the many crises and for ensuring a constant and ready supply of laboratory reagents.

- All my colleagues and friends who have provided a backbone of support and who willingly donated blood.

- Dr Peter Meissner for his experienced assistance in the final formatting and presentation.

- Andrew Jackson for unwittingly supplying the photocopier.

- My parents and Ian for their support and encouragement, always.

- This work was supported by a grant from the Medical Research Council.

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ABSTRACT

A neutral protease of the neutrophil surface: Role in the proteolysis of C-reactive protein and fibrinogen

Both of the acute phase reactants, C-reactive protein and fibrinogen, as well as neutrophils have been shown to accumulate at sites of tissue injury or inflammation. The association of C-reactive protein with neutrophils and the concomitant degradation of this ligand by a phorbol 12-myristate 13 acetate-activatable membrane-associated neutral protease has been shown in previous studies. Degradation of C-reactive protein by the neutrophil protease was shown to result in peptides with an ability to modulate various immune functions of the neutrophil. The aim of this study has been to investigate specific characteristics of the protease, with respect to cellular distribution and molecular size. The ability of this neutrophil membrane-associated protease to degrade the acute phase protein, fibrinogen was investigated. The mechanism of degradation of both C-reactive protein and fibrinogen during their association with the neutrophil was also examined.

The neutrophil protease, capable of degrading C-reactive protein, was also associated with the cytoskeleton and was proposed to be a submembrane protease localised at sites of attachment of the membrane with the cytoskeleton. The protease was found to have a molecular mass of approximately 600 kDa which, on sodium dodecyl sulphate polyacrylamide gel electrophoresis, separated into four bands which migrated to molecular mass values of 209 kDa, 316 kDa, 398 kDa and 501 kDa. This protease also possessed fibrinogenolytic activity. The fibrinogen degradation products generated by this neutrophil membrane-associated protease were distinct from the products generated by the fibrinogenolytic systems of plasmin, human neutrophil elastase and neutrophil lysosomal enzymes and were unclottable through cleavage of the Aα chain from the N-terminus and the Bβ and γ chains from the C-terminus. N-terminal cleavage of the Aα chain by the neutrophil membrane-associated protease generated the Aα1-21 peptide, previously regarded as a unique consequence of elastase activity.

Degradation of C-reactive protein and fibrinogen occurred as a result of their interaction with the neutrophil near to the CD11c integrin receptor. This interaction resulted in the egress of proteolytic activity into the extracellular medium. The fibrinogen products generated outside the cell associated with the neutrophil via the β_2 integrin receptors and the IgG Fc receptor. The interaction of the C-reactive protein degradation products with the neutrophil could not be determined. Both C-reactive protein and fibrinogen are degraded by non-stimulated neutrophils but activation with phorbol 12-myristate 13 acetate resulted in maximum degradation This upregulation of activity was achieved through activation of H7 and trifluoperazine inhibitable cellular kinases and changes in microfilament assembly.

The generation of non-clottable fibrinogen together with possible modulation of neutrophil receptor-mediated functions by the fibringen degradation products as well as the knowledge that the neutrophil protease generates C-reactive protein peptides with immunomodulatory activity implicates this neutrophil membrane-associated protease in the modulation of various inflammatory processes.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AEBSF	4-(2-aminoethyl)-benzenesulphonyl fluoride
AGP7	Azurophil granule protein 7
APR	Acute phase response
ARDS	Adult respiratory distress syndrome
ATEE	N-acetyl-L-tyrosine ethyl ester
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C	Complement
cAMP	Cyclic adenosine monophosphate
Con A	Concanavalin A
CPS	C-polysaccharide
CRP	C-reactive protein
hCRP	Human C-reactive protein
mCRP	Membrane C-reactive protein
DFP	Diisopropyl fluorophosphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetra acetic acid
ER	Endoplasmic reticulum
Fbg	Fibrinogen
FDP	Fibrinogen degradation product
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FP	Female protein (hamster)
FPA	Fibrinopeptide A
FPB	Fibrinopeptide B
GTP	Guanidine triphosphate
H7	1-(5-isoquinolinesulphonyl)-2-methylpiperazine
HBSS	Hanks balanced salt solution
H_2O_2	Hydrogen peroxide

HOCl	Hypochlorous acid
HMP	Hexose monophosphate shunt pathway
IAP	Integrin associated protein
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IMF-1	Integrin modulating factor
LAD	Leukocyte adhesion deficiency
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LRI	Leukocyte response integrin
ML-9	1-(5-chloronaphthalene-1 sulphonyl)-1H-hexahydro-1,4-diazepine
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
NEP	Neutral endopeptidase
NK	Natural killer (cell)
NLS	Nuclear localisation signal
O_2^-	Superoxide
$OH^\bullet$	Hydroxyl radical
PAF	Platelet activating factor
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PKC	Protein kinase C
Plg	Plasminogen
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PR3	Proteinase 3
SAA	Serum amyloid A protein
SAP	Serum amyloid protein

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGBP	Small GTP binding protein
SLE	Systemic lupus erythematosus
Sle^x	Sialylated lewis x
Sle^a	Sialylated lewis a
snRNP	Small ribonuclear protein
SOD	Superoxide dismutase
TCA	Trichloracetic acid
TFP	Trifluoperazine
TGF-β	Transforming growth factor β
TLCK	N-α-$_p$-tosyl-$_L$-lysine chloromethyl ketone
TNF-α	Tumour necrosis factor α
t-PA	Tissue plasminogen activator
u-PA	Urokinase plasminogen activator
VLDL	Very low density lipoprotein
α_1AT	α_1-antitrypsin
α_1PI	α_1-proteinase inhibitor
γ-IFN	γ Interferon

CHAPTER 1

INTRODUCTION

The acute phase response has been described as a complex series of events subsequent to tissue injury or infection. One such event is the alteration in the rate of synthesis and secretion of a number of so-called acute phase proteins/reactants by the liver (Schultz and Arnold, 1990; Baumann and Gauldie, 1994). This occurs within 24 hours following an inflammatory insult and is mediated by the action of mononuclear cell cytokines, including IL-1 and IL-6, on the hepatocytes (Taylor *et al.*, 1990; Schultz and Arnold, 1990; Baumann and Gauldie, 1994). In man, both C-reactive protein (CRP) and fibrinogen are acute phase proteins and their levels may increase several 1000 fold above the normal serum levels of 1 μg/ml and 4 fold above 2 mg/ml, respectively (Claus *et al.*, 1976; Yen-Watson and Kushner, 1974; Mavier *et al.*, 1990).

The precise *in vivo* function of CRP has not been elucidated but due to its remarkable phylogenetic and evolutionary conservation this role is believed to be beneficial and of importance during the inflammatory process (Pepys, 1982). Various hypotheses have been put forward in this regard based on the results of *in vitro* experimentation. For example, the ability of CRP to bind to a number of exogenous ligands, which may be liberated during the inflammatory reaction, and the subsequent activation of the classical complement pathway, points to the ability of CRP to act as an opsonin (Kaplan and Volanakis, 1974; Siegel *et al.*, 1974; Pepys, 1982). It has been suggested that clearance of these ligands from the circulation occurs via the C3b receptor as well as a CRP receptor, as yet unidentified, on both the neutrophils and mononuclear phagocytes (Buchta *et al.*, 1987; Zeller and Sullivan, 1993; Zahedi *et al.*, 1989; Tebo and Mortensen, 1990; Egenhofer *et al.*, 1993). Evidence also exists that CRP and CRP peptides, which are generated by monocytic or neutrophil-mediated proteolytic processing, can modulate certain inflammatory functions of cells important in the immune response (Dobrinich and Spagnuolo, 1991, Buchta *et al.*, 1987b; Shephard *et al.*, 1986a; Shephard *et al.*, 1988; Shephard *et al.*, 1989; Shephard *et al.*, 1990; Robey *et al.*, 1990; Tebo and Mortensen, 1991; Foldes-Filep, 1992). CRP peptides, generated by proteases associated with the neutrophil lysosomes and membranes, have been shown to modulate neutrophil chemotaxis, oxidative metabolism, degranulation and phagocytosis (Shephard *et al.*, 1986a; Shephard *et al.*, 1988; Shephard *et al.*, 1989; Shephard *et al.*, 1990; Robey *et al.*, 1987).

The inhibitory activity of these peptides was shown to be due to the inhibition of the glycolytic enzyme, enolase which results in the inhibition of glycolysis and a subsequent decrease in intracellular ATP levels (Shephard *et al.*, 1992). This may play an important role in the modification of neutrophil function at inflammatory sites thus inhibiting the tissue destructive potential of neutrophils at these sites.

CRP peptides, derived from the proteolytic activity associated with the neutrophil membrane, have been shown to be produced during association of CRP with neutrophils which have been stimulated with the phorbol ester, phorbol 12-myristate 13 acetate (PMA), at a concentration that does not release proteases from the azurophilic granules (Shephard *et al.*, 1989). The neutrophil membrane-associated protease was found to be a PMA-upregulatable enzyme which is maximally activated in the presence of CRP (Shephard *et al.*, 1989; Shephard *et al.*, 1991). Thus, CRP peptides, with the potential to modulate inflammatory functions, can be generated without intracellular processing, following the association of the intact ligand with the neutrophil surface at relatively low neutrophil activation states.

The increased hepatic synthesis of fibrinogen, in response to various inflammatory cytokines, is considered to be a necessary requirement for adequate tissue repair (Doolittle, 1981; Lau *et al.*, 1993). At an inflammatory site, fibrinogen may be converted to fibrin via the action of the serine protease, thrombin (Bailey *et al.*, 1951). Thrombin is generated from prothrombin at the final stage of a complex cascade of coagulation factor-mediated events. Tissue factor, expressed on the surface of activated endothelium and mononuclear cells in response to tumour necrosis factor and IL-1, is responsible for initiating this coagulation cascade (Edwards and Rickles, 1992). Thrombin has multi-functions including the recruitment of neutrophils to sites of inflammation. This is achieved through the ability of thrombin to upregulate endothelial expression of P-selectin, which is a glycoprotein receptor capable of interacting with specific ligands, on the neutrophil surface (Zimmerman *et al.*, 1992). This initial 'tethering' of the neutrophil to the endothelium is believed to result in further adhesion processes, mediated by the β_2 integrin receptors on the neutrophil surface and members of the immunoglobulin superfamily on the endothelial cells (Zimmerman *et al.*, 1992; Adams and Shaw, 1994). Neutrophil adhesion to the endothelium is thus strengthened and the cells are able to migrate from the vasculature toward the site of inflammation (Zimmerman *et al.*, 1992).

Once at the inflammatory site, neutrophils are believed to interact with fibrinogen via the β_2 and β_3 (leukocyte response integrin [LRI]) neutrophil integrins (Wright *et al.*, 1988; Loike *et al.*, 1991;

Gresham *et al.*, 1992). Soluble fibrinogen has also been shown to bind to the neutrophil β_2 integrin, CD11b/CD18, via the sequence Gly 190-Val 202 which resides in a 30 kDa plasmin-derived fibrinogen degradation product (Altieri *et al.*, 1993). The consequence of this association was not evaluated. The interaction of immobilised fibrinogen with neutrophils has been shown to result in the activation of neutrophil oxidative metabolism and the release of neutrophil granules with subsequent degradation of the fibrinogen by elastase (Weitz *et al.*, 1987). Fibrinogenolysis by elastase, as well as other neutrophil enzyme preparations, including cathepsin G and lysosomal enzymes, was shown to be distinct from that of plasmin (Bilezekian and Nossel, 1977; Gramse *et al.*, 1977, Plow, 1980; Weitz *et al.*, 1987; Pizzo *et al.*, 1972). The binding of soluble fibrinogen to the neutrophil LRI enhanced neutrophil Fc receptor-mediated phagocytosis (Brown *et al.*, 1990; Zhou and Brown, 1993). Studies investigating the interaction of soluble fibrinogen with neutrophils, have not evaluated possible concomitant degradation of the ligand and the influence of such neutrophil-mediated fibrinogenolysis on the accumulation of fibrin at inflammatory sites.

THE AIMS OF THIS STUDY

From previous studies investigating the biological significance of the acute phase reactant CRP, it is apparent that the generation of biologically active CRP peptides, following the interaction of CRP with a neutrophil membrane-associated protease (Shephard *et al.*, 1989), may have important consequences on the inflammatory process. It is tenable that other ligands may be degraded by this neutrophil membrane associated protease. Soluble fibrinogen has been shown to be degraded by neutrophil lysosomal enzyme preparations (Bilezikian and Nossel, 1977; Gramse *et al.*, 1977; Weitz *et al.*, 1986; Gramse *et al.*, 1980) but the ability of these proteases or proteases associated with the neutrophil membrane to act as fibrinogenolytic agents during the interaction of fibrinogen with intact neutrophils has not been investigated. The consequences of such degradation of fibrinogen may well influence the deposition of fibrin at inflammatory sites.

The aim of this study will thus include:

a) an investigation of the mechanisms involved in the neutrophil mediated processing of CRP,

b) characterisation of the membrane associated protease with respect to CRP and fibrinogen as substrates,

c) characterisation of the neutrophil protease with respect to cellular distribution and molecular size, and

d) an investigation of fibrinogenolysis by this neutrophil membrane-associated protease. The relative role of neutrophil membrane and lysosomal proteases in fibrinogenolysis during the interaction of fibrinogen with intact neutrophils will be investigated.

CHAPTER 2

BACKGROUND LITERATURE

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2.A. C-REACTIVE PROTEIN (CRP)

2.A.1. INTRODUCTION

CRP is present in trace amounts in normal plasma (±70 ng/ml in neonates and ±600 ng/ml in adults) and is described as a classical acute phase reactant in man. Following acute tissue injury, infection or inflammation hepatic synthesis of this protein occurs and its concentration may rise to as much as 1000 µg/ml within 24-48 hr (Claus *et al.*, 1976; Yen-Watson and Kushner, 1974). Serum levels of CRP have been used as an important indicator of the presence, extent and activity of disease (Pepys, 1982).

CRP is one of two members of the pentraxin family of proteins, the other being serum amyloid protein (SAP). SAP is a normal plasma glycoprotein in man and the circulating levels (30 mg/ml) are not altered by inflammatory processes (Pepys and Baltz, 1983; Dyck *et al.*, 1980; Breathnach *et al.*, 1981). CRP is composed of five identical, non-glycosylated, non-covalently associated polypeptide subunits which are arranged in a disc-like configuration with cyclic pentameric symmetry (Pepys, 1982). SAP is composed of ten identical glycosylated polypeptide subunits that are non-covalently associated in two pentameric disc-like rings which interact 'face to face' (Pepys and Baltz, 1983). The genes for both CRP and SAP are located on chromosome 1 (Floyd-Smith *et al.*, 1986) suggesting that these genes are derived from a single gene duplication event. Both proteins share approximately 60% sequence homology and both bind, calcium dependently, to specific ligands. This binding specificity is presumably related to their function although their precise biological role remains uncertain. Since both CRP and SAP exhibit highly stable evolutionary conservation it is presumed that their function is one of importance and is beneficial to the organism. Various hypotheses have been put forward regarding CRP function and these include the ability of CRP to precipitate and agglutinate its ligands e.g. foreign pathogens and toxic materials released into the circulation from dead or damaged cells and tissue. This leads to the activation of the classical complement cascade, facilitating the clearance of these ligands from plasma via phagocytic processes (Pepys, 1982). CRP has thus been described as a pro-inflammatory mediator of non-specific host resistance. CRP has also been shown to stimulate the production of various inflammatory mediators e.g. the interleukins, IL-1α, IL-1β, IL-6 and tumour necrosis factor (TNF) and conversely, has been found, at higher concentrations, to stimulate the production of the IL-1 receptor antagonist, thus assisting in the downregulation of the inflammatory effects of IL-1 (Tilg *et al.*, 1993). CRP may serve a dual role of initially amplifying the inflammatory response when, for example, a pathogen is present, and down-modulating the response when the pathogen has been eradicated. This may prevent the development of pathological inflammatory disorders.

SAP is known to associate with the amyloid fibrils during amyloidosis and is presumed to be involved in its pathogenesis (Pepys, 1982). SAP is also believed to be the predominant chromatin and DNA-binding protein thus facilitating opsonisation and subsequent clearance of the nuclear material from dead or damaged cells and consequently preventing the generation of autoimmune products (Pepys *et al.*, 1994). SAP has been shown to bind Ca^{2+} dependently to immobilised CRP and this may allow the localisation of SAP to sites of tissue damage or repair or sites where CRP is selectively deposited (Swanson *et al.*, 1992).

Recently a third member of the pentraxin family has been identified as PTX3 (Brevario *et al.*, 1992). PTX3 was cloned as an IL-1 inducible gene in endothelial cells with significantly homologous C-terminal sequences to CRP and SAP. PTX3 expression and secretion was subsequently found to be inducible in fibroblasts by IL-1 (Lee *et al.*, 1993) and in mononuclear phagocytes by lipopolysaccharide (LPS) and to a lesser extent TNF (Alles *et al.*, 1994).

2.A.2. THE ACUTE PHASE RESPONSE

Acute infections or tissue damage, together with various inflammatory stimuli, result in several dramatic humoral and cellular changes and this is referred to as the acute phase response (APR). Major changes which may occur during the APR include fever, vaso-active amine and arachidonic acid pathway-induced vascular permeability increases, neutrophilia, anaemia, changes in plasma steroids, hormones and metal ion concentrations, negative nitrogen balance and increased *de novo* synthesis and catabolism of a wide variety of proteins (Schultz and Arnold, 1990; Baumann and Gauldie, 1994). These changes are believed to promote survival during the period immediately following injury or infection, possibly resulting in the removal of infectious agents, removal of damaged tissue, protection against further damage and repair of affected organs.

The liver is the major organ involved in the APR with respect to the production of the acute phase proteins. This synthesis is the result of a co-ordinated interaction between the hepatocytes and various stimulatory cytokines e.g. IL-6, IL-1, TNF-α, Transforming growth factor β (TGF-β), γ-interferon (γ-IFN), epidermal growth factor (EGF) and leukemia inhibitory factor (Taylor *et al.*, 1990; Schultz and Arnold, 1990). The inflammatory mediators or cytokines are predominantly produced by the tissue macrophages or blood monocytes (Baumann and Gauldie, 1994). It has, however, been demonstrated that leukocytes may synthesise and release their own particular set of cytokines within the target tissue (from Baumann and Gauldie, 1994).

The serum levels of the acute phase proteins may vary considerably during the APR as some may increase while others decrease. The levels of these proteins may also vary between different species (Schultz and Arnold, 1990). It is, however, difficult to quantitate the changing levels of some acute phase reactants as an increase in serum levels may be accompanied by a parallel increase in the catabolic rate of these proteins. Acute phase proteins which increase approximately 50% above normal levels in human serum include ceruloplasmin and the complement protein C_3, while CRP and serum amyloid A protein (SAA) increase dramatically (up to 1000 fold) above normal levels (McAdam *et al.*, 1978). Fibrinogen, haptoglobin, α_1-acid glycoprotein, α_1-antitrypsin (α_1AT) and α_1-antichymotrypsin increase approximately two to four fold during the acute phase response (Mavier *et al.*, 1990). Other proteins which increase in concentration include the complement compounds C_2, C_5, C_6, C_9, factor B and total haemolytic C activity (Takahashi *et al.*, 1974 ; Bamberger *et al.*, 1978). On the other hand, the serum concentration of albumin, transferrin and α_2-H_5 glycoprotein decrease during the acute phase response and are referred to as negative acute phase reactants (Lebreton *et al.*, 1979).

2.A.3. PHYLOGENETIC CONSERVATION OF CRP

CRP demonstrates significant phylogenetic conservation. Many different species contain CRP-like molecules where CRP-likeness is evaluated mainly by its ability to specifically bind phosphorylcholine (PC) (see 2.A.6. Ligand specificity of CRP). Examples of the numerous species shown to contain CRP-like molecules include a living fossil, the horseshoe crab or *Limulus polyphemus* (Robey and Liu, 1981; Mandal *et al.*, 1991), the Japanese eel (Nonomura, 1991), the snail (Mandal *et al.*, 1991), the bony fish (plaice and the lumpsucker) (Baldo and Fletcher, 1973), *Xenopus laevis* (Seery *et al.*, 1993), rabbits (Dougherty *et al.*, 1991), chickens, mice (Patterson and Mora, 1964), hamsters (Coe *et al.*, 1981), dogs (Riley and Coleman, 1970; Yamamoto *et al.*, 1992)), horses (Takiguchi *et al.*, 1990) and monkeys (Abernethy, 1937). The guinea pig is the only species studied thus far in which CRP has not been detected (Maudsley *et al.*, 1986).

Despite various functional and broad structural similarities between CRP-like molecules of different species Ying *et al.* (1992a) have shown that a monoclonal antibody reactive with the PC binding site of human CRP (hCRP) does not react with *Limulus* or rat CRP and is 100 times less reactive with rabbit CRP. Similarly, a polyclonal antibody directed against the calcium binding site of *Limulus* CRP reacts only weakly with hCRP and rabbit CRP and not at all with rat CRP and

hamster female protein (see below). *Limulus* CRP contains the highly conserved PC and Ca^{2+} binding regions, but it demonstrates only 25% sequence homology with hCRP and exists as two trimers of similar but non-identical subunits to form a hexagonal ring structure, rather than the characteristic pentamer of CRP (Myles *et al.*, 1990). On the other hand, the mouse CRP gene has been cloned and shown to have 71% sequence similarity with the hCRP gene as well as a similar structural organisation (Ku and Mortensen, 1993).

Differences between the CRP-like molecules also exist in their acute phase behaviour where, for example, levels of *Limulus*, rat and cow CRP are constitutively high and only a marginal or no inducible response to the acute phase occurs (Pepys and Baltz, 1983) while mouse CRP is a minor plasma protein and its levels increase marginally from ±0.1 to 1 or 2 μg/ml during inflammation (Ku and Mortensen, 1993). On the other hand, hCRP (McAdam *et al.*, 1978), rabbit (Dougherty *et al.*, 1991) and horse CRP (Takiguchi *et al.*, 1990) show very low resting levels which increase dramatically following tissue injury or disease.

An interesting CRP-like molecule has been identified in female Syrian hamsters by Coe *et al.* (1981) termed hamster female protein (FP). It was so named as its synthesis appears to be under dual sex steroid and acute phase control. The resting FP levels are low in males but high in females and following an acute phase response there is a rise in the levels in the male hamsters but a drop in the FP levels of the females. Despite the ability of FP to bind PC and activate the classical complement pathway, it has since been suggested that FP is in fact the counterpart of SAP in humans, due to its localisation in amyloid deposits and its amino acid sequence homology with SAP (Tennent *et al.*, 1993; Rudnick and Dowton, 1993). It was also suggested that a true CRP counterpart, apart from FP, may exist in the hamster.

2.A.4. THE CRP GENE

The genes encoding both CRP and SAP are located on the proximal long arm of chromosome 1 between bands q12 and q23 (Floyd-Smith *et al.*, 1986). Due to their similarities in basic structure and amino acid sequence and their location on chromosome 1, it has been suggested that CRP and SAP are the products of an ancestral gene duplication event and have maintained a linkage relationship throughout evolution (Yunis and Whitehead, 1990).

The same region on chromosome 1 has also been shown to contain genes for apolipoprotein A-II, α-spectrin, peptidase C, antithrombin 3, complement factor H, CR-2 receptor, several Fc receptors, H_4 and possibly H_3 histones and selectins (Yunis and Whitehead, 1990; Bevilacqua, 1993). There appears, therefore, to exist a complex of linked genes, the products of which have immune and inflammatory associated roles as well as the capacity to interact with DNA (see 2.A.7.3. Interaction with chromatin).

The complete amino acid sequence of hCRP has been determined (Oliviera *et al.*, 1979) (Fig 1A). The mature protein contains 206 amino acids including a leader sequence of 19 amino acids which is typical of a secreted protein (Lei *et al.*, 1985). Nucleotide sequence analysis of the coding region for CRP shows it to be typical for eukaryotic genes, namely non-linear. The 5' and 3' non-coding sequences have also been determined (Lei *et al.*, 1985). Typical promoter regions described as a TATA box and CAAT box, are located 29 and 81 nucleotides upstream of the CAP site but Toniatti *et al.* (1990) have described an unusual feature of the CRP promoter region. It comprises two distinct, so-called acute phase responsive elements (APRE's) which contain the information for both liver specific/constitutive and IL-6 inducible expression. Both of these APRE's contain low affinity binding sites for the liver specific transcription factor HNF-1/LFB1 (HNF 1). Low level or constitutive expression of CRP requires weak interaction of both APRE's with HNF 1 while acute phase expression of CRP requires the interaction of the APRE's with an IL-6 inducible transcription factor H-APF-2/IL6DBF (H-APF-2). Both APRE's have been shown to contain binding sites for H-APF-2 downstream of the HNF-1 binding sites. HNF-1 thus ensures liver specific, constitutive expression of CRP whereas H-APF-2 confers acute phase, IL-6 inducible expression.

2.A.5. SYNTHESIS AND SECRETION OF CRP

It was initially believed that CRP was synthesised exclusively by hepatocytes (Hurlimann *et al.*, 1966). Since then, extrahepatic transcription of CRP has been reported in, for example, human peripheral blood lymphocytes (Kuta and Baum, 1986) and peripheral blood mononuclear cells (PBMC's) (Ikuta *et al.*, 1986) which synthesise a peptide that is recognised by anti-CRP. Murphy *et al.* (1991) also demonstrated the ability of PBMC's to transcribe the CRP gene. However, while hepatocytes express secreted CRP, peripheral blood cells express a membrane bound form of the protein (Baum *et al.*, 1983; Kuta and Baum, 1986). Kempka *et al.* (1990) initially demonstrated that CRP may be constitutively expressed as a galactose specific binding protein situated peripherally in the membrane of rat liver macrophages (mCRP).

PCA - Thr -Asp -Met -Ser -Arg -Lys -Ala -Phe -Val - Phe -Pro -Lys -Glu -Ser-	15
Asp - Thr - Ser - Tyr -Val -Ser - Leu -Lys -Ala -Pro -Leu -Thr -Lys -Pro -Leu	30
Lys - Ala - Phe -Thr -Val -Cys -Leu - His -Phe -Tyr -Thr -Glu -Leu -Ser -Ser	45
Thr - Arg - Gly -Tyr -Ser - Ile - Phe - Ser -Tyr -Ala -Thr -Lys - Arg -Gln -Asp	60
Asn - Glu - Ile -Leu - Ile - Phe -Trp -Ser -Lys -Asp - Ile - Gly -Tyr - Ser -Phe	75
Thr - Val - Gly -Gly - Ser -Glu - Ile - Leu -Phe -Glu - Val -Pro -Glu -Val -Thr	90
Val - Ala - Pro -Val - His -Ile - Cys -Thr -Ser - Trp - Glu -Ser -Ala - Ser -Gly	105
Ile - Val - Glu -Phe - Trp -Val -Asp -Gly -Lys -Pro - Arg -Val -Arg -Lys -Ser	120
Leu - Lys -Lys -Gly - Tyr -Thr -Val - Gly -Ala - Glu - Ala -Ser - Ile - Ile - Leu	135
Gly - Gln - Glu - Gln - Asp -Ser -Phe -Gly -Gly -Asn - Phe -Glu -Gly -Ser -Gln	150
Ser - Leu - Val -Gly - Asp -Ile - Gly -Asn -Val -Asn - Met -Trp -Asp -Phe -Val	165
Leu - Ser - Pro -Asp -Glu - Ile - Asn -Thr - Ile - Tyr - Leu -Gly -Gly - Pro -Phe	180
Ser - Pro - Asn -Val -Leu -Asn -Trp -Arg -Ala -Leu - Lys -Tyr -Glu - Val -Gln	195
Gly - Glu - Val - Phe -Thr -Lys - Pro -Gln -Leu -Trp -Pro - OH	206

Figure 1A. The Structure of Human C-reactive protein. The amino acid sequence of human CRP from residues 1-206 (from Fiedel, 1988)

Egenhofer *et al.* (1993) subsequently provided evidence that this membrane bound CRP was synthesised by macrophages and was not acquired externally. They also showed that this membrane form of CRP does not exist as a pentamer, as does the soluble form, but exists as subunits with neo-CRP antigenicity within the macrophage membrane. Neo-CRP has been described as an antigenically distinct form of CRP which arises under certain conditions of adsorption or modification such that the native CRP undergoes conformational alterations and results in the release of CRP subunits from the pentamer (Bray *et al.*, 1987; Potempa *et al.*, 1987). Neo-CRP may also be observed during CRP biosynthesis before aggregation of the subunits. The free subunits have reduced solubility, altered electrophoretic mobility (molecular mass 22 kDa) and unique surface characteristics due to exposed antigen epitopes which would otherwise be hidden in the pentameric configuration. The biological activity of the neo-CRP is consequently different to that of native CRP. It appears that neo-CRP preferentially activates the elements in the blood important in the inflammatory response (see 2.A.7. Biological significance of CRP) and is unable to activate the complement system. Native CRP, complexed with C-polysaccharide (CPS), on the other hand, shows preferential activity in the humoral complement system (Bray *et al.*, 1987; Potempa *et al.*, 1987). Neo-CRP is found in the acute phase plasma and is preferentially expressed in inflammatory and necrotic tissues (Bray *et al.*, 1987; Potempa *et al.*, 1987). It may be that hepatocytes synthesise and release pentameric CRP which remains inactive until it reaches sites of inflammation while peripheral blood cells at sites of inflammation might express a conformationally active form of CRP (Murphy *et al.*, 1991). This would support suggestions of CRP being involved in cell-mediated immunity.

In the case of hepatic synthesis of CRP, the first cells to produce CRP are adjacent to the portal areas. During a sustained inflammatory response cells closer to the centre of the lobules become involved (Kushner and Feldmann, 1978). This suggests the action of a circulating mediator which acts initially at the portal areas and extends into the lobule with time. It was originally thought that IL-1 was the circulating mediator solely responsible for the induction of CRP synthesis (Darlington *et al.*, 1986) but there have since been reports that IL-6 may in fact be the principle mediator (Schultz and Arnold, 1990). Much conflicting evidence has been produced in this regard, all of which seems to point to a complex co-operation between different soluble mediators. Ganapathi *et al.* (1991) as well as Smith and McDonald (1992) have demonstrated that induction of CRP in the Hep 3B cell line required the co-operative interaction of IL-1 and IL-6. This was potentiated by the synthetic corticosteroid dexamethasone and neither IL-1, IL-6 nor dexamethasone could elicit synthesis on their own. It was shown that either II -1 or IL-6 could induce CRP synthesis as long

as minimal amounts of the other cytokine were available. On the other hand Yap *et al.* (1991) and Taylor *et al.* (1990) both showed that IL-1 and IL-6 could stimulate CRP synthesis on their own using primary hepatocyte cell cultures and a hepatoma cell line, respectively. Castell *et al.* (1990) showed that in primary hepatocyte cultures only recombinant IL-6 and not IL-1β could induce CRP production which could be augmented by dexamethasone. It was suggested by Ganter *et al.* (1989) that the transcription of CRP is controlled by IL-6 and the translation by IL-1. Other inflammatory mediators have been implicated in the modification of CRP production. TNF-α has been shown to inhibit the stimulatory activity of IL-1 and IL-6 on primary hepatocyte cultures (Yap *et al.*, 1991). TGF-β was found to be a potent regulator of CRP synthesis by hepatocytes at the post-transcriptional level, but this effect was found to be very much dose dependent (Taylor *et al.*, 1990). High concentrations of TGF-β (>0,1 pg/ml) caused inhibition of CRP accumulation in NPLC/PRF/5 cells treated with both IL-1 and IL-6. Low concentrations of TGF-β, on the other hand, resulted in stimulation of CRP accumulation. Ganapathi *et al.* (1990) showed that the induction of CRP synthesis by the conditioned medium of LPS stimulated monocytes and IL-1 and IL-6 stimulated hepatoma cell lines, Hep 3B and NPLC/PRF/5, was potentiated 3-6 fold by the methylxanthine, caffeine. Yiangou *et al.* (1991) demonstrated that heavy metals, especially Hg, stimulated CRP production in mouse liver and in the Hep G2 cell line, in the presence of glucocorticoids. It has also been proposed that the production of rat CRP may be partly under hormonal control as serum estradiol-17β was shown to have an inhibitory effect on CRP production and testosterone stimulated it (Nunomura, 1990).

One may conclude from these studies that CRP production may be under varying control in cells or cell lines of differing origin and is ultimately the result of a complex interaction of a number of inflammatory mediators. Macintyre (1992) also described the upregulation of CRP production in the hepatocyte during the acute phase response to occur at a posttranslational level. This appears to occur via preferential retention or secretion of CRP from the endoplasmic reticulum (ER) due to the presence of both high and low affinity CRP binding sites in the ER of normal hepatocytes and the loss of the high affinity CRP binding site in hepatocytes under acute phase control. Consequently, the half-time for CRP secretion by the hepatocyte decreases markedly in association with increased synthesis during the acute phase response.

2.A.6. LIGAND SPECIFICITY OF CRP

The ability to bind to phosphorylcholine (PC) is considered a common functional characteristic of CRP molecules from all species (Pepys and Baltz, 1983). PC forms part of the pneumococcal CPS cell wall, is present in many other bacteria, parasites, fungi and plant extracts and is also an integral component of cells which is likely to be abnormally exposed or released from damaged tissues (Pepys, 1982; Baldo and Fletcher, 1973).

Each CRP molecule binds 2 Ca^{2+} ions and the Ca^{2+} binding site was initially believed to reside in a four amino acid acidic region within the highly conserved region between amino acids 133-147 (Swanson *et al.*, 1991) (Fig 1A). A second Ca^{2+} binding site has subsequently been identified in the amino acid residues 152-176 (Mullenix and Mortensen, 1994) (Fig 1A). Following Ca^{2+} binding these two, nearly adjacent sites, cooperate to exert a conformational change which promotes binding to PC as well as other ligands (see below).

The PC binding site on the CRP molecule is believed to reside between amino acids 47 and 63 (Fig 1A). The anion residues Lys-57 and Arg-58 are thought to bind (but not solely) to the phosphate ester while Trp-67 binds to the choline group (Swanson and Mortensen, 1990; Agrawal *et al.*, 1992). It is believed that the phosphate group of PC is critical for binding (Gotschlich, 1982).

Apart from it's PC specificity CRP can bind to a number of other ligands e.g. the polycations. These include L-lysine and L-arginine polymers, protamine, myelin basic protein, leukocyte cationic protein and histone protein (Potempa *et al.*, 1981; Shephard *et al.*, 1991; Du Clos *et al.*, 1991a). This binding is inhibited by Ca^{2+} alone but is promoted in the presence of CPS and Ca^{2+} suggesting that the polycation binding site closely approximates the PC binding site and Ca^{2+} may function as an allosteric modulator (Oliviera *et al.*, 1980; Young and Williams, 1978). Other ligands for CRP lack both phosphate and choline. CRP binds to, for example, depyruvylated type 4 polysaccharide (Heidelberger *et al.*, 1972), agarose (Pepys *et al.*, 1977) and galactose residues present in various glycans (Kempka *et al.*, 1990; Kottgen *et al.*, 1992). This binding specificity may be physiologically relevant as Kempka *et al.* (1990) demonstrated that CRP can exist as a galactose-specific binding protein in the membrane of rat liver macrophages (see 2.A.5. Synthesis and secretion of CRP). The membrane bound CRP may thus act as a receptor for mediating galactose specific endocytosis of particulate ligands at sites of inflammation. The fact that the optimum pH for binding was 6.0 further validated this theory as this pH is representative of

inflammatory sites. Binding of CRP to sugars is Ca^{2+} dependent, inhibitable by PC and is thus believed to involve the PC binding site in the CRP molecule. CRP may also bind to extracellular matrix proteins e.g. aggregated or surface bound fibronectin and laminin (Swanson *et al.*, 1989). This binding is Ca^{2+} dependent and is believed to occur via the PC binding region although Mori *et al.* (1991) indicated it to be PC independent. CRP binds to the phosholipids lecithin and sphingomyelin (Volanakis and Narkates, 1981) as well as low density lipoprotein (LDL) and traces of very low density lipoprotein (VLDL) (De Beer *et al.*, 1982).

Neo-CRP (as described above in 2.A.5. Synthesis and secretion of CRP) has distinct surface properties from the native pentameric CRP and is unable to bind PC or Ca^{2+} (Bray *et al.*, 1987).

2.A.7. BIOLOGICAL SIGNIFICANCE OF CRP

In summary, the biological significance of CRP activity includes activation of the complement cascade (Kaplan and Volanakis, 1974; Siegel *et al.*, 1974) following opsonisation of ligands through the Ca^{2+} dependent binding of CRP with PC moieties within the various CRP ligands (Mortensen and Dusciewicz, 1977). This leads to the clearance of the ligand by phagocytic cells. CRP has also been shown to lead to the direct activation of macrophages and neutrophils (Zahedi and Mortensen, 1986; Barna *et al.*, 1987; Kilpatrick and Volanakis, 1985; Zeller *et al.*, 1986a; Zeller *et al.*, 1986b).

2.A.7.1. Interaction with complement

Human CRP, aggregated with itself, CPS or other ligands, activates the classical complement (C) pathway as efficiently as the immunoglobulin IgG (Kaplan and Volanakis, 1974). Activation of C was observed preferentially with CRP ligand complexes or CRP aggregates where the native conformation of CRP and the expression of native CRP antigenicity was maintained (Jiang *et al.*, 1991). Neo-CRP (as described in 2.A.5. Synthesis and secretion of CRP) is unable to activate the complement system, as is the native, pentameric CRP.

Whereas IgG consumes all components of C, CRP only consumes 80% of the haemolytic components C1, C4 and C2 and 42-66% of C3. The membrane attack complex of C5-C9 is not consumed by CRP at all. The binding of CRP to a ligand thus results in the activation of early components of the classical complement pathway (Berman *et al.*, 1986; Osika *et al.*, 1983). The required human serum factor was found to be C1q for both CRP ligand complexes and IgG (Claus

et al., 1977), however, Jiang *et al.* (1991) demonstrated that IgG binds to the globular region (GR) of C1q and CRP binds to the collagen-like region (CLR). More specifically, CRP binds to the peptides 76-92 and 14-26 of the A chain of reduced C1q whereas IgG binds to the C chain of reduced C1q. It is believed that C1q binding and subsequent activation occurs via a conformation rather than sequence specific site on CRP (Jiang *et al.*, 1991). The optimum pH for binding is 6.3, which is within the physiological range normally found at inflammatory sites (Miyazawa and Inoue, 1990). CRP may thus undergo a pH dependent conformational change which effects its binding with C1q. Miyazawa and Inoue suggested the binding site on CRP for C to be the PC binding site but this was disputed by Jiang *et al.* (1991). This controversy may be due to the fact that the studies of Jiang *et al.* were conducted in normal human serum while those of Miyazawa and Inoue were performed *in vitro* at pH 6.3, representative of inflammatory conditions. This may imply a mechanism for the clearance of foreign and unwanted ligands at sites of inflammation. A study by Agrawal and Volanakis (1994) indicated, by site-directed mutagenesis, that the C binding site is dependent on the negative charge of Asp-112 which is unique as it appears to be modulated by the positively charged Lys-114 (Fig 1A).

2.A.7.2. Interaction with lipids

As mentioned, CRP binds to certain lipids, phosholipids as well as LDL and traces of VLDL (Volanakis and Wirtz, 1979; De Beer *et al.*, 1982). Nunomura and Hatekeyama (1990) have shown that LDL binds to CRP via the Arg residues of the PC binding site of the CRP molecule. Despite the fact that the activity of plasma lipoprotein lipase (LPL) and hepatic lipase (HL) falls during the acute phase response, LDL and VLDL triglycerides also fall during the acute phase (Sammalkorpi *et al.*, 1990). It is proposed that the binding of CRP during the acute phase to VLDL and LDL may enhance their metabolism and facilitate their removal (see 2.A.7.7. Interaction of CRP with monocytes and macrophages) during infection and inflammation. Mookerjea *et al.* (1993) suggested that the association of rat CRP with LDL results in a charge modification of the LDL molecule which leads to the stimulation of its degradation by macrophages. Substantiation for the physiological role of CRP binding to lipids was demonstrated when CRP was able to interact with liposomes containing dimyristoylphosphatidylcholine : cholesterol : galactocylceramide, resulting in complement activation of the classical pathway, followed by membrane lysis of the liposome membrane (Mold *et al.*, 1981).

2.A.7.3. Interaction with chromatin

In 1984 Robey *et al.* demonstrated the binding of CRP to the nuclei of fibroblasts damaged in culture. They also demonstrated rabbit CRP binding specifically to chromatin in the presence of Ca^{2+} with an approximate stochiometry of 1 CRP molecule/160 base pairs of DNA. They subsequently showed binding of CRP to the nucleosome core particles but these studies were done in non-physiological saline and were disproved by Du Clos *et al.* (1991a) using hCRP. Du Clos *et al.* (1991a) did, however, validate the Ca^{2+} dependent binding of hCRP to chromatin.

It was originally thought that CRP could not bind to the histones alone (Tanaka and Sueishi., 1983) but Du Clos *et al.* (1991a) demonstrated that human CRP could bind the H_3-H_4 tetramer, the H_2A-H_2B dimer as well as the individual histones H_2A, H_2B and H_1. CRP binding to H_1 was abolished upon addition of DNA, possibly indicating that binding of CRP to chromatin differs to that of binding to H_1 alone (Du Clos *et al.*, 1991a). Minota *et al.* (1993) subsequently demonstrated the presence of two CRP binding sites on the H_1 molecule. One of these CRP-binding sites was found to be approximately 20 amino acids long, Ca^{2+} independent and corresponded to the DNA-binding site of H_1. The addition of DNA might, therefore, sterically inhibit the binding of CRP to this site. The second CRP binding site on H_1 was found by Minota *et al.* (1993) to be approximately 25 amino acids in length, Ca^{2+} dependent, PC inhibitable and did not correspond to the DNA-binding region of H_1 or an autoepitope of H_1. This Ca^{2+} dependent CRP-binding site may thus represent the CRP-binding site of H_1 within the chromatin molecule. These results correlated with those of Du Clos *et al.* (1991b) who demonstrated that the Ca^{2+} dependent binding of CRP to histones was PC inhibitable and involved sequence specific residues in the C-terminal fragment of H_1. They also demonstrated a Ca^{2+} dependent, PC inhibitable CRP-binding site in the N-terminal fragment of H_2A. Shephard *et al.* (1991), however, showed Ca^{2+} dependent binding of hCRP to H_4 only.

CRP may also bind the small ribonuclearproteins (snRNP) (Du Clos, 1989) and more specifically a 70 kDa protein of snRNP (Jewell *et al.*, 1993). The hamster FP also binds histones and chromatin in a Ca^{2+} dependent and PC inhibitable manner (Saunero-Nava *et al.*, 1992).

Binding of CRP to the C-terminal fragment of H_1 is significant as this region is responsible for maintaining the compact structure of condensed chromatin (from Minota *et al.*, 1993). CRP binding could thus potentially unfold the chromatin allowing endogenous nucleases to degrade the DNA. This may be important for the clearance of nuclear material from dead or damaged cells and particularly as nuclear accumulation following cell death, can lead to the generation of autoimmune products, for example, in patients with systemic lupus erythematosus (SLE) (Minota *et al.*, 1993). Shephard *et al.* (1986b) showed enhanced micrococcal nuclear digestion following CRP binding as well as an inhibition of transcription of the damaged chromatin. It is important to note here that while CRP has been shown to bind chromatin and may play an important role *in vivo*, SAP has been demonstrated to be the main chromatin binding protein (Butler *et al.*, 1990) and also, unlike CRP and FP, may bind to naked DNA (Pepys and Butler, 1987). Despite this, Robey *et al.* (1984) claimed that the amount of CRP present was directly proportional to the amount of chromatin solubilised. They believed that this solubilisation took place via the activation of the classical complement cascade by the CRP-chromatin complex. These results were, however, disputed by Butler *et al.* (1990) who could show no activation of complement by the CRP-chromatin complex. They suggested the findings of Robey *et al.* (1984) to be due to the presence of other nuclear materials e.g. phospholipids or U1RNP, to which CRP also binds. Pepys *et al.* (1994) have subsequently claimed that in physiological conditions CRP binds only to the snRNP's and does not bind to chromatin. They showed that only SAP binds to chromatin and nucleoli *in situ* and suggested previous studies showing CRP binding to chromatin to be erroneous due to non-physiological conditions.

A major question which arises from some of the results described above is, how the CRP binds to the nuclear components as, theoretically, it would be excluded from the nucleus. Du Clos *et al.* (1990) found that CRP was structurally and functionally very similar to a well defined nuclear transport protein, nucleoplasmin. They subsequently identified a nuclear localisation signal (NLS) sequence within CRP which was capable of transporting human serum albumin (HSA) into the nucleus and also demonstrated *in vivo* nuclear localisation of CRP in intact VERO cells (a cell line of African green monkey kidney cells). SAP has also been shown to contain this NLS and is equally capable of nuclear localisation (Du Clos *et al.*, 1990). Shephard *et al.* (1991) demonstrated binding of human CRP to nuclear envelope proteins with molecular mass values of 43 kDa, 46 kDa, 52 kDa and 70 kDa. A 46 kDa envelope protein was previously implicated in the nuclear transport of RNA and may be a component of the nuclear pore complex (Sabbatini and Van Holt, 1987).

In summary, the results of these studies suggest that a function of CRP may be to clear endogenous nuclear material from dead or dying cells.

2.A.7.4. Interaction with lymphocytes

An interaction between CRP and lymphocytes was first suggested by Abernethy and Francis in 1937. Since then Hokama *et al.* (1973) have demonstrated an interaction between CRP and peripheral blood lymphocytes (PBL's) and Mortensen *et al.* (1975) showed selective binding of CRP to T-lymphocytes with subsequent inhibition of certain T-cell functions. It has since been shown, however, that purified CRP does not bind to PBL's (Ballou *et al.*, 1989) and aggregated or complexed CRP only binds to a small population of the PBL's (3-5%) (James *et al.*, 1983; Lozanski *et al.*, 1991). This binding occurs only after prolonged (up to 18 hr) incubation *in vitro*, and requires concentrations of CRP over 25 μg/ml. It would seem that CRP only binds to a specific population of lymphocytes bearing specific membrane receptors. Both James *et al.* (1982) and Muller and Fehr (1986) suggested a correlation between lymphocytes bearing IgG Fc receptors and those that bind CRP. This would present a number of possibilities: i) CRP may bind to the same receptor as IgG, ii) CRP may bind to one of the heterogeneic IgG receptors, iii) binding may occur via a distinct epitope on the Fc receptor, or iv) to a distinct receptor that is expressed primarily on IgG Fc receptor bearing cells. It has also been suggested that CRP on the lymphocyte is not acquired passively from the serum but is rather synthesised by the lymphocyte itself and expressed on the surface (Kuta and Baum, 1986; Ikuta *et al.*, 1986).

Bray *et al.* (1987) and Samberg *et al.* (1988) suggested, following a study using antibodies directed against both native CRP and neo-CRP (see 2.A.5. Synthesis and secretion of CRP), that neo-CRP, but not native CRP, is able to bind PBL's.

Not much evidence exists regarding the effects of CRP on lymphocyte function. James *et al.* (1982) showed only slightly enhanced blastogenesis of resting lymphocytes and responsiveness of allogeneic cells in a mixed lymphocyte reaction.

2.A.7.4.1. Interaction with natural killer (NK) cells

Binding of CRP to NK cells has been observed (Timonen *et al.*, 1981) although this was shown to have no affect on NK activity. CRP bearing PBL's have, however, subsequently been shown to deplete NK activity (Baum *et al.*, 1983). Khattri *et al.* (1994) have demonstrated, with the use of anti-CRP, the ability of CRP on large granular lymphocytes to modulate the NK cell lytic function. This modulation of NK cell activity by anti-CRP was achieved by altering the cytoskeletal rearrangement of the NK cells and subsequent release of lytic factors. Contrary to previous reports (Hamoudi and Baum, 1991), the modulation of NK cell activity by anti-CRP was independent of an alteration in Ca^{2+} flux.

2.A.7.5. Interaction with platelets

Initially Fiedel *et al.* (1977) obtained inconsistent results where the majority of CRP preparations (>87%) inhibited platelet activation but a limited number of CRP preparations (<13%) were not inhibitory. Subsequently, Fiedel *et al.* (1982) found the inhibition to be due to a contaminating inhibitory factor, described as LMF, with a molecular mass of 8.5-12 kDa. It was then demonstrated that LMF was in fact a degradation product of CRP (Fiedel and Gewurz, 1986). With the use of synthetic peptides Fiedel (1988) showed that the cleavage of CRP by neutral proteases of the polymorphonuclear leukocytes resulted in products with the potential to depress platelet activation. This inhibition was speculated to be due to a tuftsin sequence Gly-Lys-Pro-Arg at residues 113-116 of the CRP molecule (Fig 1A), as a synthetic peptide modelled on this sequence, described as CRP II, mimicked this inhibitory activity. CRP II has homology with the tetrapeptide Gly-Pro-Arg-Val resident in the Aα chain of fibrinogen. This tetrapeptide becomes the N-terminal aspect of the Aα chain of fibrinogen following the action of thrombin and represents the fibrin polymerisation site (see 2.B.5. Fibrin formation). It may be possible, therefore, that through sequence homology with the fibrin polymerisation site, CRP or CRP peptides may modulate platelet coagulation or clotting processes.

It has been demonstrated that neo-CRP (see 2.A.5. Synthesis and secretion of CRP) is able to induce reactions of aggregation and secretion from isolated platelets as well as to potentiate platelet activation by ADP in platelet rich plasma (PRP) (Potempa *et al.*, 1987).

2.A.7.6. Interaction with neutrophils

CRP has been shown to bind to neutrophils in a specific, saturable and rapid manner at pH 7.4 and 22°C (Buchta *et al.*, 1987a; Dobrinich and Spagnuolo, 1991; Shephard *et al.*, 1989). Binding requires Ca^{2+} and Mg^{2+} and appears to involve the PC binding site. Results from Muller and Fehr (1986) indicate approximately 30 μg/ml CRP is required for saturable binding while those of Buchta *et al.* (1987a) found that <5 μg/ml was sufficient. Of significance is the fact that this is within the range at which CRP exerts maximal effects on neutrophil function (Dobrinich and Spagnuolo, 1991). It was then suggested that there may exist both a high and a low affinity receptor for CRP on the neutrophil and that Muller and Fehr (1986) did not pick up the high affinity receptor as they did not look at binding at low enough concentrations of CRP. Buchta *et al.* (1987a) showed that aggregated IgG displaced CRP binding to the neutrophil and suggested that the CRP receptor is related to the IgG receptor. It may be, however, that the CRP receptor is physically associated with, but distinct from, the IgG Fc receptor. Zeller and Sullivan (1992)

showed that although aggregated CRP (100 μg/ml) alone had no affect on the respiratory activity of neutrophils, this form of CRP (100 μg/ml) was able to stimulate the intracellular respiratory burst of aggregated IgG-stimulated neutrophils. Zeller and Sullivan subsequently (1993) showed that the IgG FcγRII was involved in the aggregated CRP-mediated upregulation of the IgG-induced respiratory burst of the neutrophil but that this did not involve binding of CRP to the Type II Fc receptor. This may suggest that the CRP receptor is physically associated with the IgG receptor. A similar association has been suggested for the CRP receptor on macrophages and monocytes (see 2.A.7.7. Interaction with monocytes and macrophages) (Zahedi *et al.*, 1989; Tebo and Mortensen, 1990).

While examining the interaction of CRP with neutrophils Shephard *et al.* (1989) demonstrated the existence of a neutrophil membrane associated protease which is capable of cleaving CRP to smaller peptides. These CRP degradation products were found to bind to the neutrophil surface and may be responsible for the high percentages (50%) of non-specific binding encountered in many CRP/neutrophil binding studies (Muller and Fehr, 1986; Buchta *et al.*, 1987a). The low molecular mass CRP peptides (<14 kDa) generated by the neutrophil membrane associated protease were shown to inhibit neutrophil function (see 2.A.8. Proteolysis of CRP).

Although Shephard *et al.* (1986a) have shown that pure CRP had no effect on neutrophil function, other studies have demonstrated a CRP concentration effect on neutrophil function (Dobrinich and Spagnuolo, 1991). For example, at concentrations of CRP which would be expected to completely saturate the high affinity receptor (<50 μg/ml), the predominant effect appeared to be inhibition of neutrophil superoxide production. High concentrations of CRP (>100 μg/ml) led to antioxidant activity and at very low concentrations of CRP (<5 μg/ml), modest stimulation of neutrophil superoxide production was observed (Buchta *et al.*, 1987b). Chemotaxis was increased at CRP concentrations up to 1 μg/ml but higher concentrations were inhibitory (Buchta *et al.*, 1987b). Phagocytosis was enhanced by CRP at concentrations between 1 and 50 μg/ml and cyclic adenosine monophosphate (cAMP) was markedly and rapidly elevated by CRP at 50-100 μg/ml. Lysozyme release was slightly inhibited by CRP alone, at all concentrations, but specific granule release was not affected by CRP alone and was inhibited by CRP in the presence of phorbol 12-myristate 13 acetate (PMA) or Conconavalin A (Con A) (Buchta *et al.*, 1987b). This alternative inhibition and stimulation of various neutrophil functions by differing concentrations of CRP may be physiologically important as a mechanism for the prevention of 'super-activation' of the neutrophil and subsequent tissue toxicity during inflammation.

Saturation of the high affinity CRP receptor occurs at <5 μg/ml (Buchta *et al.*, 1987a) and subsequent inhibition of neutrophil function following further binding to the receptor may be due to, i) degradation of the bound CRP (Shephard *et al.*, 1989) or dissociation of the CRP receptor complex, ii) the CRP degradation products may themselves have specific biological functions and iii) binding of CRP to different high and low affinity receptors may elicit different biological responses. It has also been found that modified CRP with 'neo-CRP' epitopes (see 2.A.5. Synthesis and secretion of CRP) preferentially activates or potentiates activation of leukocytes and platelets (Potempa *et al.*, 1987; Ying *et al.*, 1992b; Zeller *et al.*, 1986a; Zeller *et al.*, 1986b). The actual mechanism for the influence of CRP on neutrophil function may be related to the phosphorylation of various proteins. Buchta *et al.* (1988) showed that neutrophils preincubated with CRP and stimulated with PMA or N-formyl-methionyl-leucyl-phenylalanine (FMLP) showed marked inhibition of phosphorylation of various proteins with molecular mass values of 43, 47, 54, 66 and 85 kDa. The extent of inhibition exceeds, however, the degree of inhibition of neutrophil functions. This is in accordance with the hypothesis that neutrophil activation may involve the activation of other pathways beside the protein kinase C (PKC) pathway (Buchta *et al.*, 1988).

2.A.7.7. Interaction with monocytes and macrophages

CRP has been shown to have an opsonic role for the phagocytic and tumoricidal functions of monocytes and macrophages (Barna *et al.*, 1984; Miyagawa *et al.*, 1988; Thomassen et al., 1993; Zeller and Sullivan, 1993; Tebo and Mortensen, 1990) and this would imply a requirement for binding of CRP to these cells. Specific binding of CRP to monocytes (Ballou *et al.*, 1989; Ballou and Cleveland, 1991; Crowell *et al.*, 1991; Zeller and Sullivan, 1993) and macrophages (Zahedi *et al.*, 1989; Nagpurker, 1993) has been observed. Binding appeared to be reversible, rapid and Ca^{2+} dependent although Zahedi *et al.* (1989) showed Ca^{2+} independent binding to macrophages, thus, apparently not involving the PC binding site. Similarly, Crowell *et al.* (1991) confirmed a Ca^{2+} independent binding of CRP to monocytes with the use of the human monocytic U937 cell line. In accordance with this controversy, Ballou *et al.* (1989) demonstrated only one binding site for CRP on the monocyte whereas others have found both a high and a low affinity receptor on both monocytes (Tebo and Mortensen, 1990) and macrophages (Zahedi *et al.*, 1989). Tebo and Mortensen (1990) showed the two CRP receptors on monocytes to have molecular mass values of 60-62 kDa and 38-41 kDa.

A CRP receptor from the macrophage was found by Zahedi *et al.* (1989) to have a molecular mass of 57-60 kDa and by Egenhofer *et al.* (1993) to have a molecular mass of 59-61 kDa. Crowell *et al.* (1991) identified a CRP receptor from U937 cells with a molecular mass identical to that of the IgG Fcγ receptor, namely 43-45 kDa. It was suggested that the high affinity receptor may be CRP specific and the low affinity receptor may be the IgG Fc receptor with a weak affinity for CRP.

There is much controversy over whether the IgG Fc receptor is a CRP receptor or not. Inhibition of CRP binding to monocytes and macrophages by IgG has been observed (Tebo and Mortensen, 1990; Crowell *et al.*, 1991; Zahedi *et al.*, 1989) but, conversely, CRP has not been shown to inhibit IgG binding. Also, in some cases inhibition of CRP binding to both monocytes and macrophages was not inhibited by IgG (Ballou *et al.*, 1989; Ballou and Cleveland, 1991; Nagpurkar, 1993). Further complications arise from the fact that there are three different types of Fcγ receptors. Monocytes possess FcγRI and FcγRII. Monoclonal antibodies against these two receptors did not effect CRP binding (Tebo and Mortensen, 1990; Crowell *et al.*, 1991; Zeller and Sullivan, 1993) but this does not exclude CRP binding to a distinct epitope on the Fc receptor. Tebo and Mortensen (1990) showed, however, that upregulation of FcγRI by IFN-γ and PMA did not affect CRP receptor expression. Despite the fact that aggregated CRP does not appear to bind to phagocytic cells via the FcγRII, it was shown that CRP potentiated the IgG mediated respiratory burst via this receptor. A monoclonal antibody against FcγRII, mAb IV.3, significantly suppressed the enhancement of IgG induced chemilluminescence by aggregated CRP but did not affect the binding of CRP (Zeller and Sullivan, 1993) (see 2.A.7.6. Interaction with neutrophils). It appears from the above studies that, as in the case of neutrophils, the CRP receptor on monocytes and macrophages is chemically distinct but possibly topographically or physically associated with the IgG Fc receptor.

An interesting observation regarding the interaction of CRP with macrophages is that of Kempka *et al.* (1990) who demonstrated the existence of CRP as a specific galactose receptor within the macrophage membrane (mCRP) (see 2.A.5. Synthesis and secretion of CRP). This mCRP was found to mediate the uptake of particulate material with exposed galactosyl groups (Kolb-Bachofen, 1991).

Following CRP binding to U937 cells, the IgG mediated respiratory burst is enhanced (Zeller and Sullivan, 1993). Barna *et al.* (1984) and Miyagawa *et al.* (1988) also demonstrated that CRP enhanced tumoricidal activity and superoxide generation in murine and guinea pig peritoneal macrophages, respectively. It is possible that the observed effects on monocyte function may have been elicited by CRP peptides as these peptides have previously been shown to lead to monocyte activation (Robey *et al.*, 1987). It has also been demonstrated that following binding of rat CRP to macrophages there is internalisation into the lysosomes and subsequent degradation (Nagpurkar *et al.*, 1993). The biological role of the peptides were not investigated. Tebo and Mortensen (1991), however, demonstrated that upon binding of CRP to U937 cells and subsequent internalisation of CRP into an endosomal compartment followed by degradation there was enhancement of the monocytes' H_2O_2 production and tumoricidal activity. Thomassen *et al.* (1993) identified a CRP peptide, described as RS-83277 (amino acids 174-185) (Fig 1A), which specifically enhanced monocyte and macrophage tumoricidal activity (see 2.A.8. Proteolysis of CRP).

As in the case of neutrophils (see 2.A.7.6. Interaction with neutrophils), neo-CRP (see 2.A.5. Synthesis and secretion of CRP) was found to potentiate the activation of monocytes and macrophages (Potempa *et al.*, 1987; Zeller *et al.*, 1986a; Zeller *et al.*, 1986b).

Cermak *et al.* (1993) have shown that CRP could induce monocytes to express tissue factor which is a potent procoagulant and initiator of the extrinsic coagulation pathway. CRP may thus play a role in altered micro-circulation in inflammatory and necrotic tissue.

A dodecapeptide within the CRP molecule (27-38) (Fig 1A) has been described as a cell binding peptide (CB-Pep) as it supports cell adhesion *in vitro* (Fernandez *et al.*, 1992). Mullenix *et al.* (1994) have identified the critical sequence within this peptide to be Phe-Thr-Val-Cys-Leu which is not similar to any of the known adhesive recognition sequences and does not mediate adhesion via the integrin receptors.

2.A.8. PROTEOLYSIS OF CRP

- Effect of CRP peptides on leukocyte function

Various studies have indicated that degradation of CRP gives rise to biologically active peptides with specific immunomodulatory activity (Shephard *et al.*, 1986a; Shephard *et al.*, 1988; Shephard *et al.*, 1990; Robey *et al.*, 1987).

It was initially described that, although native CRP could not induce phagocytic leukocytes to chemotax or produce superoxide, treatment of the purified CRP with human neutrophil derived acid proteases resulted in peptides with potent effects on leukocyte function (Shephard *et al.*, 1986a; Robey *et al.*, 1987). Robey *et al.* (1987) subsequently demonstrated that three synthetic CRP peptides containing the tuftsin-like sequences in the CRP molecule, Thr-Lys-Pro-Leu (27-30), Gly-Lys-Pro-Arg (113-116) and Thr-Lys-Pro-Gln (200-203) (Fig 1A) could stimulate phagocytic leukocyte chemotaxis and superoxide and IL-1 production. In contrast, Shephard *et al.* (1988) showed that the CRP peptides, derived from the degradation of CRP by neutrophil lysosomal enzymes, inhibited neutrophil chemotaxis, superoxide production, degranulation and phagocytosis. Discrepancies may have arisen from very slight differences in the amino acid sequences of the synthetic peptides.

Subsequent studies by Shephard *et al.* (1990) showed another three synthetic CRP peptides, generated by the action of a neutrophil membrane associated protease and corresponding to residues 201-206 or Lys-Pro-Gln-Leu-Trp-Pro (CRP III - contains a tuftsin-like sequence), 83-90 or Leu-Phe-Glu-Val-Pro-Glu-Val-Thr (CRP IV) and 77-82 or Val-Gly-Gly-Ser-Glu-Ile (CRP V) (Fig 1A), significantly inhibited neutrophil superoxide production at 50 μM. CRP III and V were also able to inhibit neutrophil chemotaxis at 50 μM. These concentrations may appear high but CRP is known to accumulate at inflammatory sites and may reach such concentrations at these sites. It may be of interest to note that CRP IV has no homology with any other known bioactive peptides. These inhibitory activities of CRP peptides may represent a significant mechanism for the prevention of 'super-activation' of neutrophils and subsequent tissue destruction at inflammatory sites. The kinetics of CRP production support this hypothesis as maximal CRP production occurs approximately 50 hours post acute phase initiating events (Shephard *et al.*, 1989). It is at this time, when host mechanisms to limit tissue destruction are required, that degradation and subsequent release of neutrophil modulating peptides would be maximal.

Thomassen *et al.* (1993) identified a CRP peptide, described as RS-83277 (amino acids 174-185) (Fig 1A), which specifically enhanced monocyte and macrophage tumoricidal activity. This CRP peptide, contained within multimellar vesicles, was later demonstrated to have immunotherapeutic activity in preclinical tumour models and especially when administered together with IL-2 (Barna *et al.*, 1993; Barna *et al.*, 1994).

Internalisation and degradation of CRP by the human monocytic cell line, U937, has been demonstrated and the peptides formed were able to activate monocytes (Tebo and Mortensen, 1991). Rat peritoneal macrophages were also shown to degrade CRP but the biological function of the peptides formed was not investigated (Nagpurkar *et al.*, 1993). Foldes-Filep (1992) showed inhibition of platelet activating factor (PAF), FMLP and PMA stimulated guinea pig macrophage superoxide production by CRP. Platelets appear to be modulated by CRP peptides but are, themselves, incapable of degrading CRP (Fiedel, 1988).

2.A.9. PROPOSED FUNCTION OF CRP

In summation, it appears that CRP has multi functions during the acute phase. Besides the ability of CRP to bind to, neutralise, detoxify and opsonise harmful or foreign substances within the circulation, an immunomodulatory role for CRP at sites of inflammation has been suggested. Vigushin *et al.* (1993), however, investigated the metabolic fate of CRP, as well as its tissue localisation in normal and diseased patients, and found that in all cases the clearance of CRP took place at a constant maximal rate. They could also not demonstrate tissue localisation of CRP which would seem to negate the hypothesis for a role of CRP at inflammatory sites.

The exact function of CRP remains a mystery as one would expect its role to be critical, due to the significant phylogenetic and evolutionary conservation of CRP. It appears, however, that the functions which have been observed to be performed by CRP are not unique to it and the guinea pig, which has no CRP-like molecule at all, does not appear to exhibit any deleterious side effects from its absence. It seems, therefore, that despite numerous studies which have demonstrated many possible functions of CRP, the search is still on to find the *raison d'etre* for its presence and preservation in all organisms, but one, studied thus far.

2.B. FIBRINOGEN

2.B.1. INTRODUCTION - FIBRINOGEN STRUCTURE

Fibrinogen (fbg) is a large, heterodimeric plasma glycoprotein which is essential for blood coagulation and platelet aggregation.

The structure of fbg was first described in 1959 by Hall and Slayter (from Doolittle, 1984). Fbg consists of three pairs of non-identical polypeptide chains (Aα, Bβ and γ) held together by 29 disulphide bonds with no free sulphydryl groups (Fig 1). The Aα chain has a molecular mass of 67 kDa (610 residues), the Bβ chain 56 kDa (461 residues) and the γ chain 47 kDa (411 residues), thus forming a molecule with a molecular mass of 340 kDa (±3000 amino acids). During biosynthesis of fbg, posttranslational modifications include glycosylation of the Bβ and γ chains, phosphorylation of the Aα chain and sulphation of the tyrosine in the γ chain (Doolittle, 1984; Mosesson, 1992). The molecule exists as a trinodular structure (of 450 °A) with two terminal, globular, carboxyl domains (D-domains) tethered to a central, N-terminal, disulphide knot (E-domain) by "coiled coil" α-helical regions. The central region of fbg contains the amino terminals of all six chains as well as 11 disulphide bonds. Three of these bonds hold the dimeric halves together, two are between the Aα and Bβ chains and the other six are thought to be situated at the junctions of the central region and the distal region and are described as disulphide rings. The distal D-domains contain the carboxyl terminal portions of the Bβ and γ chains while the carboxyl-terminal halves of the Aα chains extend out from this region and have been described as the α chain protuberances (Doolittle, 1984; Mosesson, 1992). The exact positioning of the Aα chain protuberances is still uncertain but recent studies indicate that they form part of the central globular domain (Veklich *et al.*, 1993). These α chain protuberances appear to interact intramolecularly in the fbg molecule but, with the formation of fibrin, dissociate from each other and the central domain in order that they may associate intermolecularly. The intermolecular association of the carboxyl termini of the Aα chains is known to occur during fibrin formation following the action of transglutaminase factor XIIIa (see 2.B.5. Fibrin formation) (Veklich *et al.*, 1993). Due to their lack of defined structure the α chain protuberances are desribed as random coils and due to their exposed nature are very susceptible to proteolytic attack (Farrell *et al.*, 1993).

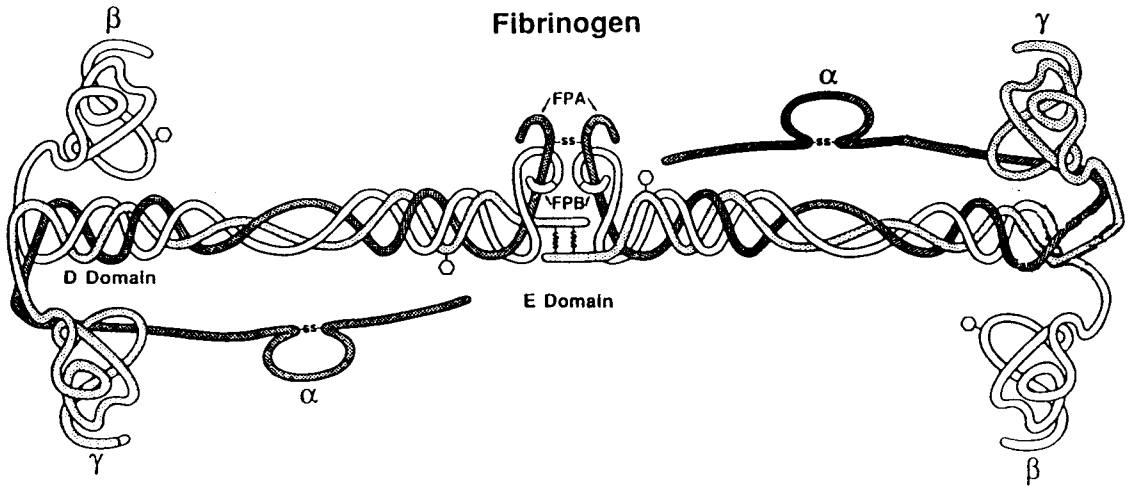

Figure 1. Schematic model of the fibrinogen molecule. Fbg consists of three pairs of polypeptide chains, Aα, Bβ and γ. The amino terminal regions of all three chains are joined in the central domain (E domain) by disuphide bonds and form a dimeric structure. This domain contains the fibrinopeptide A (FPA) and fibrinopeptide B (FPB) sequences that are cleaved by thrombin. The carboxyl termini of the Bβ and γ chains form the globular D domains, while the Aα chain extends out from this region as an α chain protuberance. The D-domains are joined to the central E domain by the 'coiled-coil' α helices (from Mosesson, 1992).

2.B.2. PHYLOGENETIC CONSERVATION OF FIBRINOGEN

All vertebrates contain similar fbg's, the most primitive being that of the lamprey, which is however, still remarkably similar to its mammalian counterparts (Doolittle, 1984).

2.B.3. THE FIBRINOGEN GENE

There is a single copy of each gene for all three fbg chains. All three of these human genes have been cloned and sequenced and the Bβ and γ chains have been found to be 33% identical and are similar enough to the Aα chain to suggest a common ancestry (from Doolittle, 1984). The homology between the chains is exemplified by the location of the cysteines.

The order of synthesis of the three gene products appears to be the Bβ chain first followed by the Aα and then the γ chain. All three chains are synthesised in a precursor form with extended amino terminal regions (signal peptides). These signal peptides are removed before or during secretion of fbg from the hepatocyte (Doolittle, 1984). Farrell *et al.* (1993) investigated the fact that the cDNA for human fbg encodes a 15 amino acid portion of the carboxyl terminus of the Aα chain which is not found in the native plasma protein. The exact function of this carboxyl extension is not known but it was found not to be involved in the assembly and secretion of fbg and removal of this extension appeared to be a normal post-translational event, although the exact means of removal

was not ascertained. Glycosylation of the γ chain occurs as an early co-translational event but Bβ chain glycosylation occurs a bit later and the Aα chain is not glycosylated at all. Phosphorylation and sulphation occur in translocational events subsequent to the ER but prior to secretion from the cell (Doolittle, 1984).

2.B.4. FIBRINOGEN BIOSYNTHESIS

Fbg is produced by the hepatocytes in the liver with its synthesis being under the overlapping control of a number of stimuli e.g. defibrination, hormones and trauma leading to an acute phase response. The release of fragments D and E, following plasmic digestion, affects the release of hepatocyte stimulating factor (HSF) from leukocytes which brings about increased fbg and acute phase protein synthesis (Doolittle, 1984).

The intracellular assembly of the three peptide chains into fbg is a stepwise process in the ER, involving a number of probable intermediates although there are conflicting reports regarding these intermediates and the pathway of assembly. A study by Roy *et al.* (1991) showed that COS-1 cells (a monkey kidney fibroblast cell line) transfected with all three fbg chain cDNA's were capable of assembling and secreting a functional fbg molecule. The predominant intermediates were Aαγ, free γ and Aα chains with AαBβ and Bβγ intermediates also being found to a lesser degree. It was proposed that surplus amounts of Aα and γ chains are maintained in the pre-golgi of the hepatocytes, either singly or complexed to each other, and production of the Bβ chain leads to the synthesis and secretion of fbg. Following production of the Bβ chain, Bβγ and AαBβ complexes are released into the rough ER where they acquire the third chain to form half-molecules. Two half molecules are then joined via disulphide bonds to form dimeric fbg. Thus, from the results of Roy *et al.* (1991), the production of the Bβ chain is a rate limiting factor in the assembly of fbg. The sequence within the Bβ chain which is critical for fbg assembly was found to be between amino acids 73 and 93, or the start of the "coiled-coil" region (Zhang and Redman, 1992). The exact function of the Aαγ intermediate is not certain as, although it is formed prior to fbg, it does not appear to be a direct precursor. In conflict with the results of Roy *et al.* (1991) a study using Hep G2 cells and transfected baby hamster kidney cells showed that Aαγ and Bβγ dimers as well as AαBβγ half molecules were formed as intermediates during the assembly and biosynthesis of fbg but disulphide-linked AαBβ complexes were not found (Huang *et al.*, 1993a). This may indicate that the interaction of the Aα and Bβ chains, which become linked via three disulphide bonds, may only occur later on in the assembly of fbg. Thus, the disulphide bonds between the Aα and Bβ

chains most likely only occur when AαBβγ half molecules are formed between Aαγ and Bβγ intermediates and/or when AαBβγ half molecules dimerise to generate the whole molecule. Doolittle (1984), on the other hand, suggested the formation of a homotrimer γ_3 followed by stepwise displacement of two of the γ chains and subsequent formation of the heterotrimer intermediates Aαγ2 and Bβγ2. In this way a pool of γ chains is generated. Conflicting results may be explained by the observation of Oddoux and Greininger (1994) that differences in fibrinogen assembly may be species specific. For example, they found that in chicken hepatocytes, the Aα chain plays a critical role in fbg assembly, rather than the Bβ chain in human hepatocytes.

A common feature of all the studies described above is the observation that the dimerisation of two half molecules, via disulphide bonds, is one of the final steps in the assembly of fbg with the disulphide bonds in the amino terminal region of the Aα and γ chains holding the two AαBβγ half-molecules together (Huang *et al.*, 1993b). For this, the Aα28-Cys residue interacts with the same residue on the other Aα chain, and the cysteine residues at positions 8 and 9 in the γ chain pair with their counterparts in an anti-parallel or reciprocal fashion. It has been suggested that this would allow the dimeric half-molecules to orientate in an anti-parallel manner (from Mosesson, 1992). A bond between Aα36-Cys and Bβ65-Cys of the other half molecule also appears to be critical for linking the two AαBβγ half-molecules together (Huang *et al.*, 1993b). The disulphide rings which flank the "coiled-coil" region may also play an important role in dimer assembly (Zhang and Redman, 1994). These rings may be necessary for maintaining the "coiled-coil" structure and may be critical in aligning the four cysteines of the amino terminus of one half molecule with the corresponding four cysteines of the other half molecule, thus promoting disulphide linkage and dimer formation. The residues involved in the formation of these disulphide rings at the amino terminus include Bβ76-Cys, Bβ80-Cys, γ19-Cys and γ23-Cys (Zhang and Redman, 1994). The disulphide rings at the carboxyl terminus of the "coiled-coil' are believed to be important for secretion of fbg. It was suggested that there is a co-operative effect where some of the disulphide bonds may influence the formation of other disulphide bonds on different domains of the molecule (Zhang and Redman, 1994).

An interesting observation derived from these studies (Huang *et al.*, 1993a; Roy *et al.*, 1991) was that fbg assembly, intracellular transportation and secretion does not require the hepatocyte machinery and it was suggested that the information for these functions may be inherent in the fbg chains themselves.

2.B.5. FIBRIN FORMATION

Blood clotting is a complex series of events involving many sequential cascades of zymogen activation where a serine protease, thrombin, ultimately converts fbg into an insoluble cross-linked fibril network, described as fibrin (from Esmon, 1993). Thrombin elicits the release of fibrinopeptide A (FPA, Aα1-16) and then fibrinopeptide B (FPB, Bβ1-14) from the Aα and Bβ chains of fbg, respectively (Fig 1) (Bailey *et al.*, 1951). Spontaneous polymerisation between fbg molecules occurs through electrostatic interactions of the newly positively charged central domains with the slightly negatively charged terminal domains (see 2.B.5.1. Polymerisation sites). Different polymerisation paths may develop depending on the relative rates of release of FPA and FPB (Doolittle, 1984). Initially, two stranded protofibrils (trimers and tetramers) are formed in a half staggered overlap conformation. Following protofibril formation, end to end cross-linking occurs and the formation of fibrin polymers with γ dimers is observed. Crosslinking of the γ chains is initiated by the plasma transglutaminase factor XIIIa and gives rise to a covalent bond between the two γ chains providing structural stability and integrity to an otherwise easily deformable clot (from Mosesson, 1992). Aα chain crosslinks between the carboxyl termini are also observed, during prolonged clot formation, and are also initiated by the transglutaminase factor XIIIa (Veklich *et al.*, 1993). Crosslinks may occur between Aα and γ chains and are initiated by human erythrocyte transglutaminase (Murthy *et al.*, 1991).

Release of FPB occurs more slowly than the release of FPA and gives rise to lateral associations and branching. The coarseness of a clot appears to relate to the release of FPB and, more importantly, a delay in release of FPB (after protofibril formation) appears to be necessary for normal fibre assembly with lateral branching (Weisel *et al.*, 1993). Removal of FPA thus allows the formation of linear polymers, which are described as fibrin I or desA fibrin, whereas the delayed release of FPB allows lateral involvement and the formation of thick fibres, described as fibrin II or desAB fibrin, which is more resistant to proteolysis (Doolittle, 1984).

2.B.5.1. Polymerisation sites

The active site of thrombin consists of two separate domains, namely the catalytic site and an extended fbg recognition sequence (FRS). Aα7-16 of fbg interacts with the catalytic site of thrombin and residues Aα27-50 are involved in binding the FRS of thrombin (Martin *et al.*, 1992). The release of the second FPA from the fbg molecule by thrombin occurs 16 times faster than that of the first and is suggested to be due to a co-operative effect of thrombin which is assisted by the

close orientation of the amino terminals of the two Aα chains within the fbg molecule (from Mosesson, 1992). Less is known of the interaction of the Bβ chain with thrombin although it has been suggested that Bβ15-42 may interact with the FRS (Binnie and Lord, 1993).

Removal of FPA from the central amino terminal domain uncovers the peptide sequence Gly-Pro-Arg (G-P-R) which interacts, Ca^{2+} and conformation dependently, with the carboxyl terminal residues γ357-373 (Laudano and Doolittle, 1981; Cierniewski and Budzynski, 1993; Shimuzu *et al.*, 1992). A critical residue within this sequence was found to be the Tyr of γ363 (Yamazumi and Doolittle, 1992). Peptides beginning with the sequence G-P-R, or containing the G-P-R sequence only, are very effective inhibitors of fibrin polymerisation (Laudano and Doolittle, 1980; Kawasaki *et al.*, 1993). Slight variations in the peptide sequence may, however, alter its affinity e.g. G-P-R-V (which is the actual sequence in fbg) has a much reduced affinity for the carboxyl region of the γ chain when compared with G-P-R-P (Laudano and Doolittle, 1980).

Release of FPB exposes the sequence Gly-His-Arg (G-H-R) which interacts Ca^{2+} dependently with the distal domain of the Bβ chain of another fbg molecule (Laudano and Doolittle, 1981). Peptides beginning with the sequence G-H-R do not, however, inhibit fibrin formation although, G-P-R and G-H-R in combination, offer the most effective inhibition of clotting under physiological conditions with thrombin (Laudano and Doolittle, 1981).

Formation of the γ-γ cross-link involves reciprocal binding of a donor Lys at position 406 and an acceptor Gly at the carboxyl termini of the respective γ chains, at position 398 or predominantly 399 (Purves, 1988).

2.B.6. INTERACTIONS OF FIBRINOGEN

Fibrinogen has been shown to interact with a number of ligand substrates and cells with important physiological consequences.

2.B.6.1. Interaction of fibrinogen with plasminogen

Plasminogen (plg) binds to fbg via lysine side chains on the fbg molecule and this has been indicated to be important for plg activation (Yonekawa *et al.*, 1992; Gron *et al.*, 1992) (see 2.B.7. Fibrinogenolysis).

2.B.6.2. Interaction of fibrinogen with thrombin

Interaction of thrombin with fbg leads to the release of FPA and FPB and subsequent clot formation (see above).

2.B.6.3. Interaction of fibrinogen with calcium

There are three high and 10-12 low affinity Ca^{2+} binding sites on the fbg molecule. Two of the high affinity binding sites are found on the D-domain (Fig 1) while no high affinity binding sites were found on the E-domain (Lindsey *et al.*, 1978). One of the Ca^{2+} binding sites involved in clot formation is on the γ chain between residues 303 and 356 (Varadi and Scheraga, 1986). Removal of the tightly bound Ca^{2+} renders fbg more susceptible to thermal and alkali danaturation and plasmin digestion. In the absence of Ca^{2+} fibrin formation is much slower and the rigidity of the resultant clot is much diminished (from Hermans and McDonagh, 1982). Ca^{2+} binding may upregulate the affinity of a polymerisation site on the Bβ chain following removal of the FPB and is also essential for the binding of transglutaminase factor XIIIa to fbg (Laudano and Doolittle, 1981).

2.B.6.4. Interaction of fibrinogen with leukocytes

Fbg has been shown to bind rapidly, Ca^{2+} dependently and reversibly to neutrophils and purified monocytes but not lymphocytes (from Trezzini *et al.*, 1988). This association is generally believed to occur via the β_2-integrin which is ubiquitously expressed on all leukocytes of lymphoid and myelo-monocytic lineage and which may recognise multiple ligands (see 2.C.7.3. The integrins).

The interaction of fbg with leukocytes has pathophysiologic consequences. Intravascular accumulation of leukocytes may severely contribute to the onset and development of atherothrombotic lesions while local activation of coagulation and fbg deposition on the leukocyte surface invariably contributes to thrombus organisation in the atherosclerotic plaque (from Altieri *et al.*, 1993). On the other hand, fbg association with leukocytes may play a role in specific mechanisms of host defence and inflammation. This is indicated by the ability of fbg binding to CD11/CD18 and the leukocyte response integrin (LRI, see 2.C.7.4.1. Neutrophil integrins) to modulate monocyte oxidative responses (Trezzini *et al.*, 1988), Fc-mediated phagocytosis of complement-opsonised particles (Gresham *et al.*, 1989), neutrophil chemotaxis (Senior *et al.*, 1992) and hydrogen peroxide (H_2O_2) production by neutrophils in the presence of stimulators,

TNF and PMA (Nathan *et al.*, 1989; Zhou and Brown, 1993). Binding of fbg via the γ chain C-terminal sequence, Lys-Gln-Ala-Gly-Asp-Val (K-Q-A-G-D-V) , to the CD11b/CD18 receptor on monocytes has been shown to decrease monocyte induced lymphocyte responsiveness (Robson *et al.*, 1994). Leukocyte interactions with fbg may also play a role in the localisation and diapedesis of monocytes and neutrophils in the extravascular compartment as a result of a collaborative network of neutrophil interactions with endothelial cells and endothelial associated fbg (from Altieri *et al.*, 1988, see 2.C.7.1. Endothelial-neutrophil interaction). The binding of fbg to the monocyte has also been implicated in the differentiation of monocytes into macrophages (Trezzini *et al.*, 1988).

2.B.6.5. Interaction of fibrinogen with platelets

Platelets bind to and contain fbg, as well as other factors involved in clot formation, and the overall phenomenon of normal haemostasis depends on their mutual interaction (Bennett *et al.*, 1983). In particular, fbg enhances the agonist induced platelet aggregation response and platelets in turn cause the fibrin clot to retract (Bennett *et al.*, 1983). Fbg binds to platelets via the β_3 integrin of the cytoadhesin family, namely the $\alpha IIb\beta_3$ receptor, or more commonly the gpIIb/IIIa glycoprotein complex (see 2.C.7.3. The integrins) (Bennett *et al.*, 1983). Like fbg binding to the β_2 integrins, binding to the β_3 integrin is divalent cation dependent (Peerschke, 1993). The platelet receptor must be activated in order to bind soluble fbg and this is achieved with adenosine diphosphate (ADP) or thrombin, although the binding of small fbg peptides to $\alpha IIb\beta_3$ does not require activation (Tomiyama *et al.*, 1992). This may indicate a conformational change in the receptor, following activation, thus allowing access for the binding of macromolecules. The binding of fbg to the gpIIb/IIIa complex thus leads to the bridging of these receptors between adjacent platelets as well as receptor modification, neoantigen exposure and subsequent signal transduction giving rise to intracellular second messengers and the ultimate result of platelet adhesion and aggregation (Perutelli and Mori, 1992).

It was initially believed that the binding site within fbg for the gpIIb/IIIa complex, lay in the carboxyl terminal 12 residues of the γ chain and more specifically in the K-Q-A-G-D-V sequence within γ401-γ411 (Kloczewiak *et al.*, 1984). It was subsequently shown that the Arg-Gly-Asp or R-G-D sequence at the carboxyl terminus (Aα572-575, -R-G-D-S) and in the middle of the Aα chain of fbg (Aα95-98, -R-G-D-F) is also involved in the binding of fbg to stimulated platelets. This was indicated by the ability of this sequence, in a tetrapeptide R-G-D-X, to inhibit agonist

induced platelet aggregation by competitive inhibition of fbg binding (Gartner and Bennett, 1985; Hantgan *et al.*, 1992). The conformation of the tetrapeptide appears to correlate directly with its inhibitory activity (Peishoff *et al.*, 1992). The K-Q-A-G-D-V peptide is also capable of inhibiting platelet aggregation but to a lesser degree than R-G-D-S (Hantgan *et al.*, 1992; Xinjie *et al.*, 1993) although Kloczewiack *et al.* (1984) claimed that K-Q-A-G-D-V was in fact the more potent inhibitor. The differences observed may have been due to variations in the conformations of the prepared peptides. Of interest is the fact that K-Q-A-G-D-V crosslinks with IIb (residues 256-306) while R-G-D peptides crosslink to both IIb and IIIa (IIIa, residues 109-171) (from Perutelli and Mori, 1992; Sheu *et al.*, 1992).

The binding described above is reversible, Ca^{2+} dependent and involves soluble fbg. Parise *et al.* (1993) have, however, documented an irreversible, non-covalent and Ca^{2+} independent binding of immobilised fbg to purified gpIIb/IIIa via novel binding sites on both the platelet receptor and the fbg molecule. This may be explained by the exposure of novel binding sites on immobilised fbg or fibrin due to altered conformation and these exposed sites could act co-operatively resulting in a high affinity interaction with distinct properties. In conflict with the above results, is the observation by Muller *et al.* (1993) that soluble fbg binds to the platelet in a biphasic manner with initial weak, reversible binding followed by a stronger, irreversible interaction. The method used to measure this binding involved incorporating the $\alpha IIb\beta_3$ integrin into planar lipid bilayers and thus avoided possible artefactual results of previous methods with immobilised receptor on plastic surfaces or interference from other receptors when platelets were used. The results of this report may have implications for many previous studies where inhibition of fbg binding to platelets by various peptides was assessed. The period of preincubation of these peptides with platelets may have influenced the resultant inhibition observed.

2.B.6.6. Interaction of fibrinogen with bacteria

Fbg may clump certain strains of *Staphylococcus aureus* but the exact function of this interaction is not known and not all species have fbg binding receptors capable of this interaction. Fbg binding to *S. Aureus* occurs via the same region on the γ chain where fbg/platelet interactions are believed to occur (Doolittle, 1984).

2.B.7. FIBRINOGENOLYSIS

2.B.7.1. Digestion of fibrinogen by plasmin

Fbg is important for haemostasis and wound healing but it is necessary that it eventually be cleared from sites of injury and this is achieved primarily via the plasmin mediated pathway. Fibrin dissolution is also essential for the removal of clots which may be found in the general circulation (Castellino, 1981; Pizzo *et al.*, 1972; Doolittle, 1981; Lau *et al.*, 1993). Fibrinogenolysis does not appear to differ significantly from fibrinolysis indicating that the intermolecular associations of fibrin do not shield the molecule from proteolytic attack (Ferguson *et al.*, 1975).

A crucial step in the digestion of fbg is the activation of the inactive proenzyme, plg to active plasmin by tissue-type plasminogen activator (t-PA), which is released from vascular endothelium (Yonekawa *et al.*, 1992). It has been shown that fibrin monomers, polymers as well as fibrin (not crosslinked) and to a lesser degree, fbg, may themselves enhance the rate of plasmin formation (Gron *et al.*, 1992). Yonekawa *et al.* (1992) have also shown that plasmin derived fragments of fbg may accelerate t-PA catalysed plg activation. This has been attributed to a peptide in the Aα chain described as FCB-2 (Aα148-160) (Voskuilen, 1987) and a peptide in the γ chain described as FCB-5 (γ311-379) (Yonekawa *et al.*, 1992). FCB-5 exerts its effect by interacting lysine independently with t-PA while FCB-2 interacts with plg, in a lysine dependent, as well as independent, fashion (Grailhe *et al.*, 1994). FCB-2 and FCB-5 have a particular orientation with respect to each other in the fibrin molecule and may this may serve to concentrate both t-PA and plg on the fibrin surface (Yonekawa *et al.*, 1992). These enhancing sequences appear to be exposed upon fbg fragmentation or fibrin formation.

Plasmin has a specificity for lysyl peptide bonds (Plow, 1980). Digestion of both fbg and fibrin by plasmin gives rise to a set of core products, described as A-E, which have molecular masses of 85kDa or less (Pizzo *et al.*, 1972) and these very specific products have been used to assess and characterise fbg structure (Fig 2). The Aα chain of fbg is the first to be degraded by plasmin and is cleaved from the carboxyl terminus. Concomitant with the degradation of the Aα chain is the formation of fragments A, B, C (±15 kDa) and X (240-265 kDa). The Bβ chain is the next to be degraded, from the amino terminus (Ferguson *et al.*, 1975). When approximately half of the Bβ chain has been cleaved to a species with a molecular mass of 52 kDa, described as $B\beta^1$, fragment X is present in maximum amounts (Doolittle, 1984). Fragment X appears to exist in a number of

variants, with differing molecular masses, relative to the extent of degradation of the Aα and the Bβ chains. The two Aα chains of the dimer may also degrade at different rates (Azpiazu and Chapman, 1992). The γ chain is the most resistant to degradation and concomitant with its cleavage is the formation of fragments D (83-100 kDa), E (±41 kDa) and Y (±155kDa). Both fragments D and E are terminal digestion products while fragment Y is transient (Doolittle, 1984). Like fragment X, fragment Y may exist in several intermediate forms depending on the extent of degradation of the γ chain e.g. D_1 (92 kDa), D_2 (86 kDa) and D_3 (82 kDa) which differ in the extent of the carboxyl terminal degradation of the γ chain from 38 kDa to 32 kDa and 28 kDa respectively (Ferguson *et al.*, 1975). If the γ chain is crosslinked (184 kDa), then no further digestion of the γ chain occurs and a product described as DD is formed. The cleaved Bβ chain in fragment Y has a molecular mass of 44 kDa, is described as $B\beta^{11}$, and is resistant to further degradation along with the $A\alpha^{11}$ chain (12 kDa) (Azpiazu and Chapman, 1992). Fragment D contains partially degraded Bβ and γ chains and extensively degraded Aα chains. Fragment D also contains different species of cleaved γ chain described as γ^{1} (± 44kDa, D_1), γ^{11} (±37 kDa, D_2) and γ^{111} (±25 kDa, D_3) and may exist as distinct populations with differing amounts of these different constituent chains (Pizzo *et al.*, 1972). Fragment E contains extensively degraded Aα, Bβ and γ chains with molecular masses of 10 kDa and less and these are linked by disulphide bonds.

Degradation products with extensively degraded Aα chains still retain the ability to clot but as the Bβ and γ chains are degraded this ability is lost (Pizzo *et al.*, 1972). Fragment X, for example, retains the ability to clot despite extensive degradation of the Aα chain and slight degradation of the Bβ chain. As the γ chain begins to degrade with the advent of fragment Y formation, the products still have the ability to clot but clotting times become much extended. Properly formed fragment Y cannot clot at all (Pizzo *et al.*, 1972). It is interesting to note that although fragment D_1 (γ86-411) cannot clot it still retains complementary sites for polymerisation (γ363) and is able to act as an inhibitor of fibrin formation. Fragments D_2 (γ86-356) and D_3 (γ86-301), on the other hand, do not contain polymerisation sites and do not exhibit this anticoagulant activity (Yamazumi and Doolittle, 1992). Fragment E has also lost these sites and can neither clot nor act as an anticoagulant (Azpiazu and Chapman, 1992; Varadi and Scheraga, 1986).

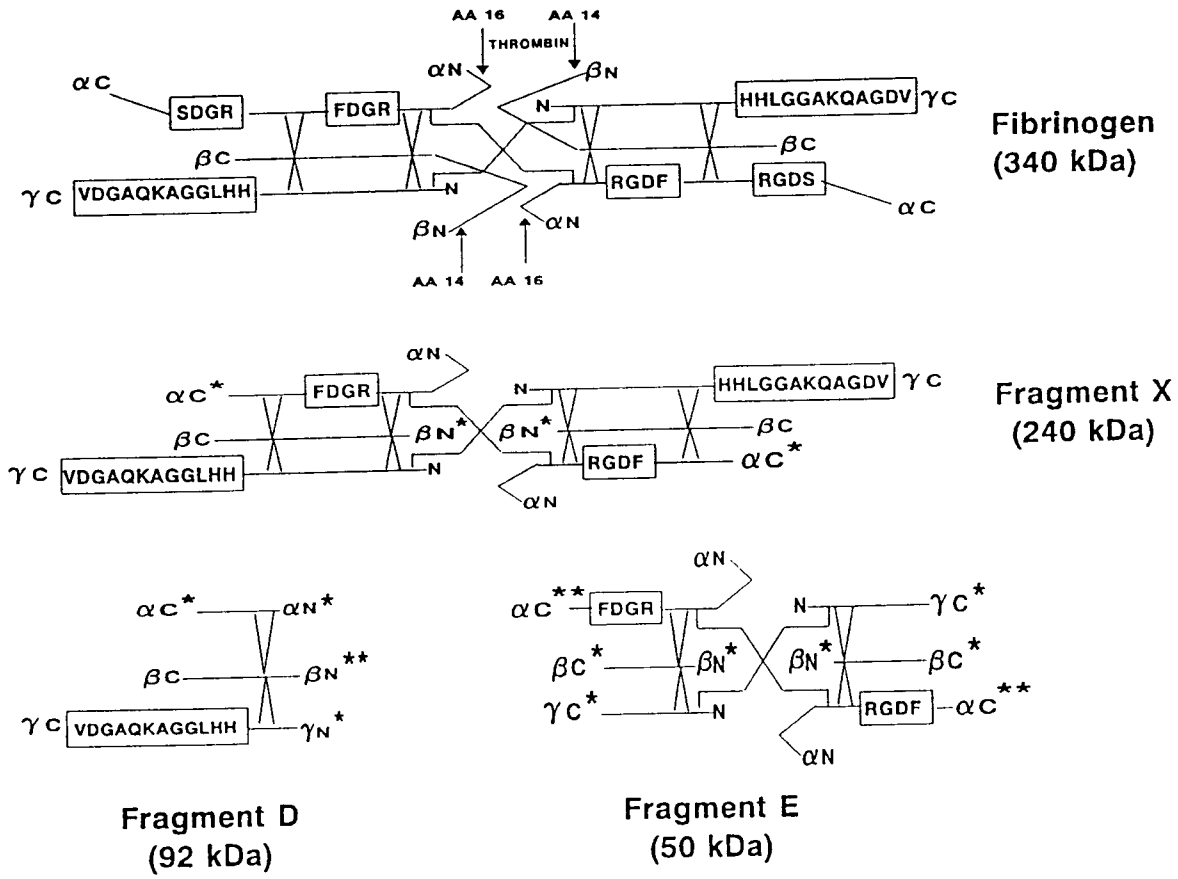

Figure 2. Schematic form of fibrinogen. Each half molecule of fibrinogen contains three non-identical chains which are covalently linked by disulphide bonds and are joined by symmetrical disulphide bonds between two Aα and two γ chains (Doolittle, 1984). Three globular domains exist: one central E domain, containing the N-termini of the six chains, and two peripheral D domains, containing the C-termini (Doolittle, 1984). Fragments X, D and E are plasmic products. * denotes new termini generated through initial plasmin cleavage and ** denotes new termini generated through further plasmin cleavage. The arrows indicate the cleavage sites of thrombin on the Aα and the Bβ chain, resulting in the generation of fibrinopeptides A and B. The γ chain C-terminal dodecapeptide sequence (γ400-411), which contains a crosslinking site, is indicated in the box.

2.B.7.2. Digestion of fibrinogen by non-plasmin mediated pathways

In addition to the plasmin mediated fibrinogenolytic systems in the plasma phase of the blood there exists a quantitatively important (70-80%) cellular-phase component to blood fibrinogenolysis (Langleben and Moroz, 1985; Hammouda and Moroz, 1987; Grau and Moroz, 1989). Neutrophils are known to accumulate intra- and extravascularly at fbg deposits and are capable of penetrating thrombi and exerting anticoagulant effects, contributing significantly to the percentage of blood fibrinolysis (Moroz, 1984; Plow, 1982). Cellular (neutrophils and possibly monocytes) fibrinogenolytic activity may be stimulated by physiological stimuli such as venous occlusion, exercise, pathological processes such as surgical trauma and thromboembolism, and pharmacological agents such as urokinase and t-PA. This cellular activity may be reduced in certain surgical patients and patients with SLE (Langleben and Moroz, 1985; Grau and Moroz, 1989).

Bilezikian and Nossel (1977) degraded fbg with a leukocyte extract and after a certain time the fbg was rendered unclottable. They showed, with the use of inhibitors, that the major enzyme responsible for this fibrinogenolytic activity was human neutrophil elastase (HNE). Gramse *et al.* (1977) characterised the degradation products of fbg following the action of HNE and found them to be distinct from those generated by plasmin, although there were some antigenic similarities between D and E and products generated by elastase. A major difference lies in the observation that plasminolysis gives rise to specific end products whereas cleavage by HNE is progressive and the extent of degradation is directly related to HNE concentration. Another difference lies in the ability of elastase to readily cleave all three fbg chains whereas the γ chain is only cleaved after prolonged incubation of fbg with plasmin. Elastase has a specificity for alanyl peptide bonds and its activity results in the release of fibrinopeptide A (FPA) and fibrinopeptide B (FPB) containing fragments whereas plasmin cleaves no FPA containing fragment until the end stages of digestion (Plow, 1980; Bilezikian and Nossel, 1977).

Both plasma and interstitial spaces contain numerous elastase inhibitors (Travis and Salvesan, 1983). Despite this, stimulated neutrophils are able to maintain HNE in an active form (Weiss and Regiani, 1984). The reason for this has been suggested to be that elastase mediated fibrinogenolysis occurs in a 'pocket' on the neutrophil membrane surface where fbg is bound to the Mac-1 receptor (see 2.C.7.4.1. Neutrophil integrins) (Weitz *et al.*, 1987). The secreted elastase is thus protected from the high molecular mass protease inhibitors present in the surrounding medium e.g. α_2-macroglobulins and α_1-proteinase inhibitors (α_1PI's). This mechanism requires adherence of

neutrophils to a ligand or ligand coated surface which results in a protected zone that excludes inhibitors with a molecular mass of >50 kDa. Consequently, cell associated proteases are more resistant to inhibition than free enzyme. It was also suggested that HNE activity may be protected from antiprotease activity by the antioxidants produced by the neutrophil (oxidative inactivation of α_1PI) (Weiss, 1989; Bangalore and Travis, 1994) but this was claimed not to be so under physiological conditions due to the presence of anti-oxidants and competitive antagonists (Campbell *et al.*, 1982). Another suggestion was made by Bangalore and Travis (1994) who have demonstrated that upon secretion of HNE from the activated neutrophil, there is a partial rebinding of the enzyme to the neutrophil surface. They found this 'rebound' form of elastase to be reistant to inhibition by the elastase-specific inhibitors.

The initial high molecular mass products, formed through the action of elastase (270 kDa, larger than fragment X), are no longer clottable by thrombin, indicating that degradation is not confined to the carboxyl termini of the Aα and the Bβ chains, as is that of plasmin (Plow, 1980). Recent work by Bach-Gansmo *et al.* (1994), however, showed that the initial cleavage by HNE occurs at the carboxyl terminus of fbg and this cleavage appears to cause the prolongation of clotting time and loss of clottability. The low molecular mass fbg products of elastase activity differ in size to all other known fbg products and exhibit potent anticoagulant activity (Plow and Edgington, 1975). A specific low molecular mass, FPA containing peptide is produced by the early action of elastase on fbg and is formed by the cleavage of the Aα chain between Aα21-Val and Aα22-Glu (Weitz *et al.*, 1986). This peptide (Aα1-21) has since been used as a specific indicator for elastase activity and a detection assay was developed to demonstrate elevated levels of Aα1-21 in patients with α_1AT deficiency and pulmonary emphysema (Weitz *et al.*, 1992).

An important observation by Weitz *et al.* (1987) showed that unstimulated neutrophils in contact with fbg coated filters still released small quantities of Aα1-21. This would suggest that the interaction of the cells with the substrate was, in itself, enough of a stimulus for HNE release. This may explain the presence of Aα1-21 in the plasma of normal individuals where each day $\pm 10^9$ neutrophils emigrate from the vascular compartment into extravascular tissues (Weitz *et al.*, 1987) and in doing so must come into contact with the extracellular matrix comprised of, for example, fbg. A low level of fibrinogenolysis may thus be an inescapable consequence of neutrophil emigration from the vasculature.

Cathepsin G has also been indicated to play a major role in the leukocyte fibrinogenolytic pathway (Plow, 1980). Cathepsin G, within the neutrophil granules, initially cleaves the Aα chain in the presence of $CaCl_2$, followed by the Bβ and then the γ chain (Gramse *et al.*, 1980). Cathepsin G has a specificity for leucyl bonds and aromatic amino acids and the products formed are not identical to those produced by the action of plasmin but a fragment with similar antigenicity and molecular mass to fragment E is formed (Plow, 1980). Ca^{2+} appears to have a significant effect on fbg degradation by cathepsin G, where the γ chain shows a greater susceptibility to cathepsin G than the Bβ chain in the absence of Ca^{2+}. Also, the main degradation products appear smaller in molecular mass than those formed in the presence of Ca^{2+}. In the case of plasmin, only the susceptibility of fragment D is affected by the presence of Ca^{2+}.

Platelets have been shown to contain proteolytic enzymes capable of digesting fbg but they appear to contribute a quantitatively minor role to fibrinogenolysis in the circulation (Moroz, 1984).

Macrophages also contain enzymes with elastolytic activity, capable of cleaving fbg, but these enzymes are immunologically unrelated to neutrophil elastase (Plow, 1982).

Monocytes have been shown to bind fbg via the CD11b/CD18 (Mac-1) receptor (see 2.C.7.3. The integrins), followed by internalisation into the lysosomes and subsequent degradation (Simon *et al.*, 1993).

2.B.7.3. Fibrinogen degradation products (FDP's) - Function

Fragments D, DD and E are typically present in patients with disseminated intravascular coagulation (DIC), which is commonly associated with severe bacterial infection and neutropenia, as well as related conditions in which neutrophil defence against bacterial infection may be compromised (Kazura *et al.*, 1989; Robson *et al.*, 1994). Kazura *et al.* (1989) showed that FDP's inhibit neutrophil O_2^- release and chemotaxis stimulated by FMLP. Fragment E was primarily responsible for the inhibition of FMLP induced neutrophil O_2^- release, possibly by inhibiting FMLP interaction with neutrophil receptors. Inhibition of chemotaxis by FDP's also involves cell receptor/FMLP modulation. PMA and zymosan activated neutrophil O_2^- release was also inhibited by FDP's, but inhibition was not due to interference of stimulator binding and was possibly due to impairment of activation and translocation of PKC from the cytosol to the membrane. D and DD

were also shown to inhibit lymphocyte proliferation in response to T-cell mitogens, allogeneic mononuclear leukocytes and anti-CD3 *in vitro* (Robson *et al.*, 1993). This inhibitory activity is derived from a sequence within the C-terminus of the γ chain of D or DD namely K-Q-A-G-D-V which binds to the CD11b/CD18 monocyte receptor (see 2.C.7.3. The integrins) (Gresham *et al.*, 1992). Conflicting results to these were reported by Hatzfield *et al.* (1982) who showed stimulation of proliferation of human haemopoeitic cells (monocytes, promyelocytes, pre-B cells, T-cells and erythroid cells) by FDP's *in vitro*.

Other proposed functions of FDP's include the following:
a) Fragment D has been shown to induce cytoskeletal alterations and eventual retraction of venous endothelial cells *in vitro*. Ge *et al.* (1992) showed that this endothelial cell detachment occurred concurrently with an increase in cell associated urokinase plasminogen activator (u-PA) and, to a lesser extent, t-PA and a subsequent increase in sub-endothelial matrix (fbg and vitronectin) degradation by plasmin. Fragment E was also found to elicit endothelial cell detachment but to a much lesser extent. This function may have pathological relevance where elevated levels of D are found in patients with adult respiratory distress syndrome (ARDS) associated with increased pulmonary vascular permeability and injury. These results were, however, in direct contrast with those of Tanaka and Sueishi (1993) who found that fibrin caused endothelial cell injury while fragments D and E did not. The exact reason for this discrepancy is not certain. b) Fragment E has been found to stimulate focal smooth-muscle cell proliferation during the formation of stenosing atherosclerotic lesions. The E fragment must, however, have been derived from fibrin and thus have lost the fibrinopeptides in order to exert these effects. Fbg derived fragment E or fbg did not stimulate muscle cell proliferation (Stirk *et al.*, 1993). c) Fragment E competes with fbg for fibroblast binding and inhibits directed migration of these cells in a fbg concentration gradient (from Kazura *et al.*, 1989). d) Fragments D and E appear to be chemotactic for monocytes (from Lorenzet *et al.*, 1992). e) Fragments X and D have been shown to adhere to melanoma cells, *in vitro*, via the $\alpha_V\beta_3$ integrin (see 2.C.7.3. The integrins) (Felding-Habermann *et al.*, 1992). Whereas the binding of whole fbg to melanoma cells results in significant cell spreading, the binding of fragment X and D does not. This differing biological response following the binding of fragment X and D to the β_3 integrin, in comparison to binding of whole fbg, appears to relate to the different relative affinities of these ligands for the same receptor (Felding-Habermann *et al.*, 1992). f) Various low molecular mass peptides (<20 kDa) have been found to induce chemotaxis of neutrophils as well as to inhibit lymphocyte blastogenesis (from Kazura *et al.*, 1989; Robson *et al.*,

1993). Low molecular mass FDP's are also able to stimulate the release of endothelial cell-derived growth factors (Lorenzet *et al.*, 1992). FPB has been shown to have chemotactic activity for neutrophils with a potency equivalent to that of C5a, leukotrine B_4 and FMLP (from Kazura *et al.*, 1989). g) Bβ1-42 mediates retraction of bovine artery endothelial cells and h) Bβ43-47 induces vasodilation of bovine mesenteric arteries (from Lorenzet *et al.*, 1992). i) Synthetic peptides containing the carboxyl terminus of the γ chain, specifically γ402-411 containing the K-Q-A-G-D-V sequence, were found to inhibit the binding of fbg to the platelet surface via the $\alpha_{IIb}\beta_3$ (gpIIb/IIIa) integrin receptor (see 2.C.7.3. The integrins and 2.B.6.5. Interaction of fbg with platelets) (Kloczewiak *et al.*, 1984). Synthetic peptides containing the R-G-D sequence, present in the Aα chain of fbg, and retaining an appropriate conformation, were, however, found to be more potent inhibitors of platelet aggregation than the K-Q-A-G-D-V sequence (Xinjie *et al.*, 1993). j) Synthetic peptides beginning with, or containing only, the sequence Gly-Pro-Arg (G-P-R), which represents the amino terminal segment of the fbg Aα chain after the release of FPA, can prevent or delay the clotting of fbg by thrombin (Laudano and Doolittle, 1980; Kawasaki *et al.*, 1993). This anticoagulant activity was observed to be due to the interaction of the peptide with fbg and not thrombin. The exact sequence of the peptide was also found to be critical e.g. G-P-R-P, was shown to be a far more efficient anticoagulant than G-P-R-V (Laudano and Doolittle, 1980). Anticoagulant activity has also been observed in a 24 kDa product of plasmic digestion of fbg which represents the middle two thirds of the Aα chain beginning at Met-240 from the N terminus (Lau, 1993). The exact mechanism of inhibition of fibrin polymerisation by this 24 kDa peptide is not yet certain but results point to an interaction with fbg, rather than with thrombin or Ca^{2+} ions. It was suggested that this peptide does not function as a complementary binding site for fibrin monomers per se, but may interfere with the maintenance of polymerisation (Lau, 1993).

These observations suggest that increases in the plasma concentration of various fbg fragments modulate the inflammatory response by virtue of the effects these fbg fragments have on multiple cellular components of blood.

2.C. THE NEUTROPHIL

2.C.1. INTRODUCTION - THE ROLE OF THE NEUTROPHIL IN INFLAMMATION

Neutrophils represent over 90% of the circulating granulocytes. They typically contain numerous granules which can be observed at the ultrastructural level. These granules fuse with the phagosomes, following phagocytosis, to form phagolysosomes (Roitt *et al.*, 1985). Initially believed to be a purely mechanical phagocytic cell for the removal of foreign substances from the body, the neutrophil has recently come to the fore as a highly sophisticated inflammatory cell that may interact with its environment and perform numerous inflammatory functions.

Acute inflammation is a complex response of the body to local injury or infection. Neutrophils represent the first line of defence during inflammation and rapidly emigrate from the vasculature to the site of injury and infection. This is achieved in a five step process involving attachment to the blood vessel wall, transmigration into the tissues, chemotaxis, phagocytosis and killing (from Malech and Gallin, 1987) (Fig 3). Each of these cellular processes is initiated by the occupation of specific surface receptors. Once phagocytosed the foreign particle is treated to a lethal 'cocktail' of reactive oxygen species, acidic pH, cationic proteins and a number of antimicrobial proteases. Although this represents a very effective means of host defence aberrant neutrophil numbers or behaviour may potentiate severe pathophysiological disorders and it is, therefore, necessary that we understand the mechanism of neutrophil function (from Malech and Gallin, 1987).

In this review a general outline of neutrophil function will be provided while particular relevant aspects will be discussed in detail.

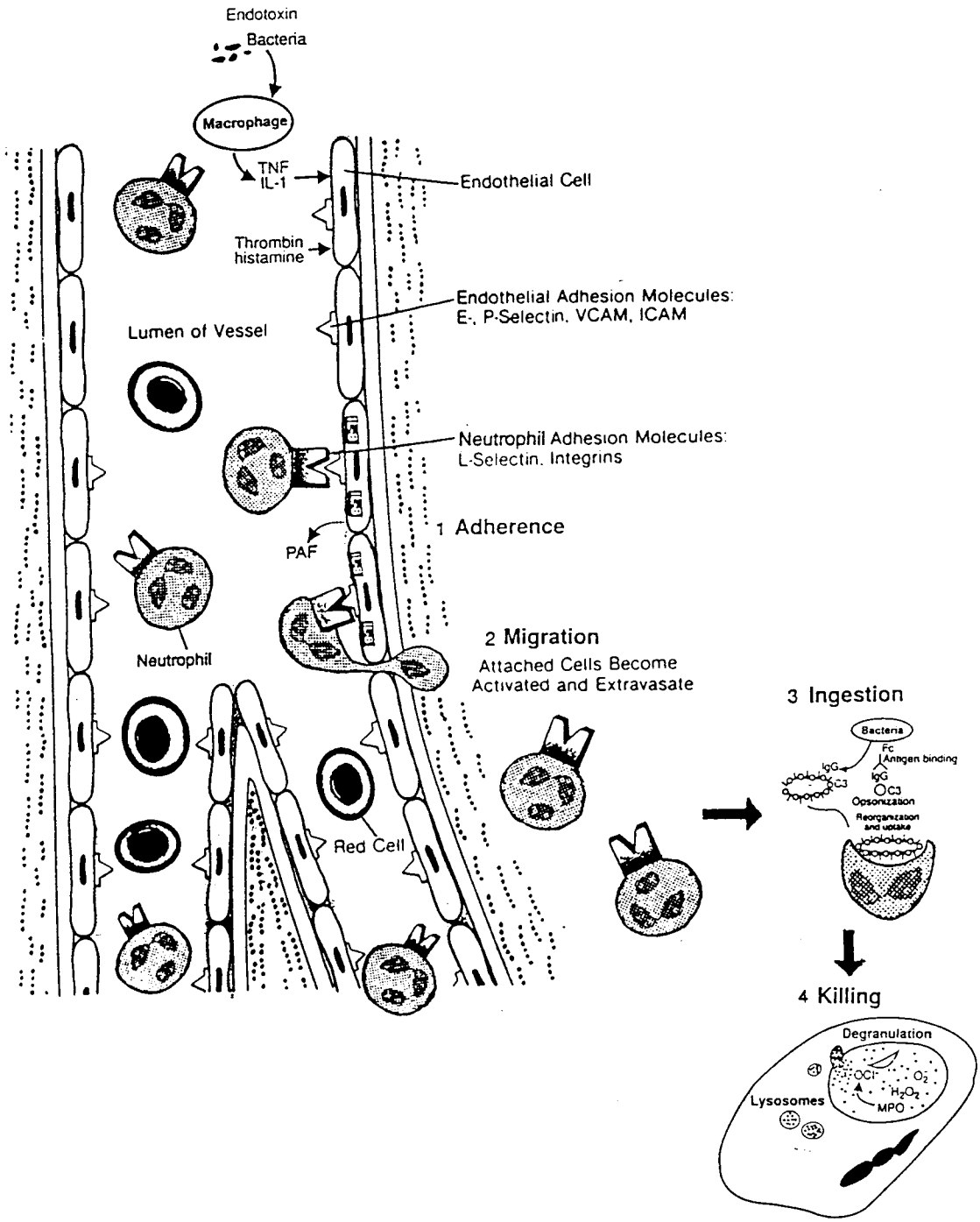

Figure 3. Migration of neutrophils out of the vasculature and into the underlying tissue, toward sites of infection or inflammation, occurs as a co-ordinated sequence of events. Initially the neutrophils, activated by various inflammatory mediators, 'roll' along and sample the endothelial surface. This rolling action is mediated by the selectins, on both the neutrophil and the endothelium, and their respective oligosaccharide ligands. Expression of IL-8 and release of PAF by the endothelial cells stimulates expression of the β_2-integrins on the neutrophil which may then interact with counter-receptors of the immunoglobulin superfamily on the endothelium. This leads to 'tight' adhesion of the neutrophil to the vessel wall and is accompanied by a change of shape of the neutrophil followed by extravasation of the cell into the underlying tissue. The neutrophil migrates toward the site of infection as its movement is directed along a chemotactic gradient. Opsonisation of the foreign particle by IgG and C3 allows phagocytosis to proceed by means of the IgG Fc and C3 receptors present on the neutrophil. The foreign particle becomes incorporated into the phagosome where it is treated to a lethal cocktail of reactive oxygen species, acidic pH, cationic proteins and a number of antimicrobial proteases (adapted from Gearing and Newman, 1993 and Cohen, 1994).

2.C.2. NEUTROPHIL CHEMOTAXIS

When an acute inflammatory response develops at a local site, there is an increase in vascular permeability and subsequent migration of the neutrophils out of the vasculature toward the infected site. The mechanism of diapedesis is discussed in detail below (see 2.C.7.4. Endothelial-neutrophil interaction).

Chemotaxis is a complicated and finely regulated process involving the interaction of chemotactic factors, their antagonists and their receptors and results in the orientated migration of neutrophils as they migrate along a chemotactic gradient (Van der Valk and Herman, 1987). Chemotactic substances are numerous and include various host plasma-derived factors as well as substances released from various host cell types and infective organisms (Table 1). The most important chemotactic agents are believed to be the complement components, C3 and C5a, as well as components and degradation products of the clotting system (Van der Valk and Herman, 1987; Wardle, 1986).

Table 1. Factors that are chemotactic for neutrophils (Wardle, 1986).

ENDOGENOUS	
Plasma derived	•Immune complexes •C5a and C5a desArg complement components •Kallikrein (by activation of coagulation) •Leucegresin (or protease treated immunoglobulin) •IgG •Oxidised lipids or platelet factor 4 •Fibrinopeptides and FDP
Cell-derived	•Lymphokines (from lymphocytes) •Neutrophil chemotactic factor (PAF, HETE or LTB4) of other neutrophils, mast cells or monocytes •Collagen fragments in damaged tissues
EXOGENOUS	
	•Chemotactic peptides and lipids of bacteria •Calcium Ionophores chemotactic peptides FMLP, Con A: PMA, opsonised zymosan (C3b): combined C3b and IgG stimulation

Receptors on the neutrophil responsible for directional chemotaxis include those for N-formyl peptides of bacterial origin e.g. FMLP, C5a, IL-8 and bioactive lipid products e.g. PAF and leukotriene B4 (from Cohen, 1994). Occupancy of a critical number of receptors leads to rapid shape changes (polarisation) and directional movement of the cell. The most extensively studied of the chemotactic receptors are those for FMLP (from Cohen, 1994). The expression of the FMLP receptors has been shown to be increased by stimulation of the neutrophil with low doses of PMA which leads to the limited and preferential release of the specific granules (see 2.C.5. Neutrophil granules) (Fletcher *et al.*, 1982). Following ligand binding to the FMLP receptor, the mechanisms for signal transduction are unclear but may generate multifunctional signals e.g. chemotaxis, degranulation and increased oxygen uptake, depending on the concentration of the stimulus (from Cohen, 1994).

2.C.3. NEUTROPHIL PHAGOCYTOSIS

Once the neutrophil has migrated to the source of the chemotactic stimulus e.g. an invading pathogen, it ingests the foreign particle in a process described as phagocytosis (Borregaard, 1988; Stossel, 1994). Phagocytosis is mediated by the IgG Fc and C3 surface receptors and, following receptor occupation, an invagination occurs on the neutrophil surface as actin/myosin polymerisation alters the organisation of the underlying microfilaments (Fig 3). The neutrophil changes shape and 'fingers' of organelle-free cytoplasm, described as pseudopods, extend out and surround the foreign particle (Stossel, 1994). The plasma membrane engulfs the foreign particle and as the pseudopods meet and fuse, the foreign object is encased in a phagocytic vacuole (Borregaard, 1988). The primary or azurophilic granules fuse with the newly formed phagocytic vacuole and release their toxic components resulting in the destruction of the foreign body by various means (see 2.C.4. Neutrophil killing).

As mentioned briefly, the receptors involved in the phagocytic process are the C3 and Fc receptors. The Fc receptors include FcRII and FcRIII and both recognise the Fc portion of the immunoglobulin. Ingestion of bacteria mediated by the FcRII has been noted to effect the microbicidal response more efficiently than FcRIII (Kimberly *et al.*, 1990; Huizinga *et al.*, 1990). The complement receptor CR1 is primarily responsible for the recognition of C3b but, in some cases, ingestion of C3b-coated particles requires concomitant occupancy of the Fc receptor (Joiner *et al.*, 1984). CR3 mediates the binding of C3bi-coated particles but also requires the second Fc-mediated signal for ingestion (Joiner *et al.*, 1984).

2.C.4. NEUTROPHIL KILLING

Following phagocytosis of a foreign pathogen a number of destructive substances or processes are released or activated in the phagocytic vacuole and in concert these ensure a highly effective means of 'killing' (Fig 3) (Wardle, 1986; Cohen, 1994). The toxic substances may, in some instances e.g. adhesion and spreading, also be secreted from the granules, into the extracellular milieu and may bring about the degradation of extracellular matrix components. This may be beneficial for the cell in the case of neutrophil migration from the vasculature toward inflammatory sites, but may also be contributory to the development of tissue destructive pathological disorders (from Suchard and Boxer, 1994).

2.C.4.1. The respiratory burst

Phagocytosis or adherence of neutrophils to extracellular matrix proteins may be accompanied by a sudden increase in O_2 consumption (from Cohen, 1994; Sud'ina *et al.*, 1991). This leads to the oxidation of increased amounts of glucose (via the hexose monophosphate shunt, HMP) and the generation of reactive oxygen intermediates e.g. superoxide (O_2^-) (Clark, 1990; Rotrosen *et al.*, 1992). O_2^- is generated by the activation of membrane NADPH oxidase by cytochrome b558 which is released from the secondary granules (see 2.C.5. Neutrophil granules). Hydrogen peroxide (H_2O_2) is then generated either by the spontaneous dismutation of O_2^- or via a superoxide dismutase (SOD) catalysed reaction. Secondary oxygen metabolites may also be formed (Wardle, 1986; from Cohen, 1994). Hypochlorous acid (HOCl), which is primarily responsible for killing bacteria in the phagosomes, is formed by the action of myeloperoxidase (MPO) on H_2O_2. MPO, in the presence of H_2O_2, may also lead to the deamination and decarboxylation of amino acids to generate chloramines which cause oxidative damage to proteins (Weiss *et al.*, 1983). Hydroxyl radicals ($OH^{\cdot}$) are formed by the interaction of HOCl with O_2^- as well as the reduction of Fe^{3+} to Fe^{2+} by O_2^- resulting in reduction of H_2O_2 to $OH^{\cdot}$ (Ramos *et al.*, 1992). An interesting observation is the ability of lactoferrin to bind Fe^{3+}, preventing the formation of $OH^{\cdot}$ and possibly presenting a mechanism for the control of hydroxyl radical formation at inflammatory sites (Britigan *et al.*, 1986).

The respiratory burst is localised at the membrane of the cell and consequently also of the phagocytic vacuole membrane resulting in the release of toxic oxygen metabolites into both the extracellular medium and the phagosome (from Van der Valk and Herman, 1987). Pre-treatment of neutrophils with endotoxin e.g. LPS as well as TNF, has been shown to 'prime' the neutrophil to release increased amounts of superoxide when stimulated and this appears to be mediated by an alteration in calcium flux (Bajaj *et al.*, 1992; from Cohen, 1994).

2.C.4.2. Acidification of the phagosome

Some microbes are killed by low pH and some of the neutrophil antimicrobial systems require a low pH for optimal activity e.g. the MPO dependent formation of HOCl and bacterial killing by HOCl are optimal at pH 5.0 (Hurst and Barrette, 1989). In resting cells acidification results from release of lactic acid formed during neutrophil aerobic glycolysis. In phagocytosing cells numerous factors result in acidification. These include release of lysosomal granule hydrolases into the phagosome, generation of protons by internalised micro-organisms and their degradation products, dissociation of carbonic acid formed through the HMP and the active transport of H^+ ions through a Na^+/H^+ antiport and an anion/OH^- ion antiport (from Cohen, 1994).

2.C.4.3. Antimicrobial proteins and proteases

The antimicrobial proteins and proteases work in concert with the free radicals (see above) and are all contained within the numerous cytoplasmic granules of the neutrophil (from Cohen, 1994). The neutrophil granules and their toxic contents are discussed in detail below.

2.C.5. NEUTROPHIL GRANULES

The neutrophil granules contain severely toxic products and it is crucial that they not be released by the unstimulated circulating neutrophil (Borregaard *et al.*, 1993b). It seems that the neutrophil has a highly sophisticated means of communicating with the environment and is capable of separately mobilising four different types of intracellular granules and secretory vesicles, some of which are crucial for interaction with the endothelium and migration, others for phagocytosis and microbial killing (Borregaard *et al.*, 1993b). The process of degranulation is guanidine triphosphate (GTP)-dependent and the presence of small GTP-binding proteins (SGBP's) have been suggested to play a role in the differential exocytosis of the different granules. Two of these SGBP's have thus far been identified and specifically associate with the membrane of the secondary granules or the plasma membrane. They have been named rap1 and rap2 and are notably not associated with the cytosol or the primary granules (Maridonneau-Parin and De Gunzburg, 1992).

Upregulation of the neutrophil surface membrane proteins has been shown to be the result of the exocytosis of different granule subsets and may lead to the expression of, for example, adhesion proteins, receptors for complement factors and chemotactic peptides and components of the microbial NADPH-oxidase, necessary for respiratory burst activity (Borregaard *et al.*, 1993b). Thus, upregulation of the surface membrane proteins may represent an important feature of the inflammatory response of the neutrophil.

The toxic components of the granules may be confined to the phagolysosomes following phagocytosis or may be released into the extracellular medium during adhesion and spreading of the neutrophil. In many instances this release of destructive substances from the cell may lead to a variety of pathologic processes (from Suchard and Boxer, 1994). The process of neutrophil mediated endothelial injury is thought to result in the loss of the selective permeability barrier giving rise to oedema and haemorrhage, as well as the loss of nonthrombogenic surface properties resulting in platelet adhesion and coagulation. Culminatively these effects appear to play a role in the pathophysiology of, for example, ARDS, vasculitides (Kawasaki syndrome, Wegeners granulomatosis) as well as the complications arising from clinical treatments such as thrombolytic therapy of myocardial infarction and haemodialysis (pulmonary leukostasis, microembolism, disseminated intravascular coagulation) (Westlin and Gimbrone, 1993).

Neutrophil granules are traditionally classified into the peroxidase-positive (azurophil or primary) and peroxidase negative (specific or secondary) granules and more recently the tertiary or gelatinase granules (Kjeldson *et al.*, 1992) and the secretory vesicles (Borregaard *et al.*, 1987, 1990; Sengelov *et al.*, 1993). These groups may be even further differentiated according to density and listed in order of decreasing density are, a) the dense peroxidase positive granules, rich in defensins, b) light peroxidase positive granules, low in defensins, c) dense peroxidase negative granules, containing lactoferrin but no gelatinase, d) intermediate peroxidase negative granules, containing lactoferrin and gelatinase and e) light peroxidase negative granules, containing gelatinase but no lactoferrin (Borregaard *et al.*, 1993a).

The primary or azurophilic granules are formed at the myeloblast stage, are characterised by the presence of MPO and are the stores for most of the proteolytic and bactericidal proteinases including elastase, the cathepsins, lysozyme and the recently identified proteinase 3 (PR3) (Borregaard *et al.*, 1993a). MPO is important for the generation of reactive oxygen species as it

acts on H_2O_2 and converts it to the toxic HOCl (see 2.C.4.1. The respiratory burst). The membrane of the azurophil granules does not contain any important inflammatory receptors and they are mobilised very slowly indicating their primary function to be digestion and killing of phagocytosed material. These granules also act late in the inflammatory response upon disintegration of the cell (Borregaard *et al.*, 1993a).

Specific granules are formed at the myelocyte stage, are unique to neutrophils and serve as a marker for terminal myeloid maturation. They are characterised by lactoferrin and vitamin B_{12}-binding protein (Borregaard *et al.*, 1993a). The specific granule also contains numerous membrane proteins, namely cytochrome b_{558}, FMLP receptors, TNF receptors, G-proteins, Mac-1 and many others. They also contain the proteases, collagenase, lysozyme and plasminogen activator. A recent study (Suchard and Boxer, 1994) demonstrated that the exocytosis of the specific granules is a pre-requisite for the production of the toxic reactive oxygen derivative, H_2O_2 and they postulated that this was related to the delivery of cytochrome b to the plasma membrane leading to the activation of NADPH oxidase. The specific granules have also been described as the 'adhesomes' (Singer *et al.*, 1989) as they were believed to be responsible for the initiation of the inflammatory response in the neutrophils, due to their content of membrane adhesion receptors. Subsequently, however, the secretory vesicles, which are more easily mobilisable and contain inflammatory receptors, are considered to be more important than the specific granules for the initiation of inflammatory processes (Sengelov *et al.*, 1993).

The tertiary granules are a sub-set of light peroxidase negative granules which contain gelatinase but no lactoferrin. Although most lactoferrin containing granules contain gelatinase they should not be confused with the tertiary granules which contain the majority of the total cell gelatinase despite their low number (approximately 25% of peroxidase negative granules) (Kjeldson *et al.*, 1992; Kjeldson *et al.*, 1993). It was suggested that the tertiary ganules may contribute to the upregulation of the neutrophil membrane proteins during inflammation as they contain the same membrane components as the specific granules and are more readily mobilised. Tertiary granule numbers are now thought to be insufficient to account for the total upregulation of the membrane proteins during the inflammatory response. Their mobilisation does, however, appear to account for the substantial release of gelatinase from the neutrophil (Borregaard *et al.*, 1993a). A major difference between tertiary and specific granule gelatinase is the association of this protease with a 25 kDa neutrophil associated gelatinase lipocalin (NGAL) in the specific granules and the absence of this association in the tertiary granules (Borregaard *et al.*, 1993a).

The secretory vesicles have been described as highly mobilisable light vesicular structures containing alkaline phosphotase on the luminal side of the vesical membrane (Borregaard *et al.*, 1993a; Borregaard *et al.*, 1993b). These vesicles contain plasma proteins not synthesised by the cell and therefore represent a specialised form of endocytic vesicle which, once formed, behave like granules. They are suggested to be responsible for the upregulation of surface membrane proteins, namely Mac-1 (see 2.C.7.3.1. Neutrophil Integrins), following their fusion with the plasma membrane during exocytosis, and may thus be responsible for regulating the interactions of neutrophils with the endothelium during the inflammatory response (see 2.C.7.4. Endothelial-neutrophil interaction) (from Borregaard *et al.*, 1993a; Borregaard *et al.*, 1993b).

The secretory vesicles are the most easily mobilisable and secreted granules, followed by the tertiary, gelatinase-rich granules, then the specific and finally the azurophilic granules (Borregaard *et al.*, 1993a). With a given stimulus the granules 'hook on' to the microtubule system and are subject to the mechanical force of the microtubules. The lighter and smaller the granule, the faster it will move (Borregaard *et al.*, 1993a). Granule exocytosis also appears to involve the sequential release of granule components with some being specifically released before others (Csernok *et al.*, 1994). The mechanism for this specificity or the signal which allows incorporation of a particular granule constituent into the plasma membrane e.g. PR3, where the other constituents are released into the extracellular milieu, e.g. elastase and cathepsin G, is not known.

2.C.6. NEUTROPHIL PROTEASES

The predominant function of neutrophil proteases is to facilitate neutrophil migration through the basement membrane as it moves out of the vasculature to inflammatory sites (from Adams and Shaw, 1994) and to digest foreign organisms within the phagolysosome (from Suchard and Boxer, 1994). The neutrophil contains primarily two groups of proteases, the metalloproteases and the serine proteases.

2.C.6.1. Neutrophil matrix metalloproteinases (MMP)

MMP's mediate the degradation of the extracellular matrix and basement membrane components, collagen, glycoproteins and proteoglycans (Hirose *et al.*, 1993). The metalloprotease gene family consists of at least two distinct interstitial collagenases (MMP-1 or fibroblast collagenase and MMP-8 or neutrophil collagenase), three types of stromelysins, putative metalloproteinase 1 and two gelatinases (72 kDa [MMP-2] and 92 kDa [MMP-9]). All of these enzymes contain an essential catalytic zinc-binding domain, an amino terminal domain containing a "cysteine switch",

which preserves the latent state of the enzyme, and a variable carboxyl terminus (Hirose *et al.*, 1993). They are all secreted as zymogens which undergo extracellular activation by, for example, elastase and reactive oxygen species. Apart from zinc the MMP's also require Ca^{2+} for activity. Interstitial collagenase and gelatinase are the two MMP's produced by the neutrophil (Hirose *et al.*, 1993).

2.C.6.1.1. Collagenase

Human neutrophil collagenase (MMP-8) is stored as a glycosylated proenzyme within the specific granules and is secreted in response to various inflammatory mediators (from Knauper *et al.*, 1993). Activation of the procollagenase occurs in the extracellular medium following the cleavage of at least 80 or 81 N-terminal amino acids following interaction with proteases, reactive oxygen species or mercurial compounds (from Knauper *et al.*, 1993). MMP-8 has a specificity for the interstitial collagens. The most distinctive characteristic of interstitial collagenases is their ability to cleave the interstitial collagens at a single peptide bond. The substrate specificity of this enzyme appears to reside in a 62 amino acid carboxyl terminal region and is influenced by a disulphide loop in this region. Another locus, preceding the 62 amino acid collagenase specific locus, also appears to be necessary for general catalytic activity. Asp-253 in this region was found to be critical for activity and may represent a possible divalent cation binding site (Hirose *et al.*, 1993).

2.C.6.1.2. Gelatinase

Gelatinase exists as a 92 kDa protein (MMP-9) in the specific granules or a 72 kDa (MMP-2) form in the tertiary granules (see 2.C.5. Neutrophil granules; Borregaard *et al.*, 1993). Unlike the neutrophil collagenases, the gelatinase gene appears to be identical in neutrophils, fibrosarcoma cells, macrophages, monocytes etc. despite the fact that it is stored in granules in the neutrophils and is constitutively secreted by all other granulocytes (Devarajan *et al.*, 1992). Gelatinase is co-ordinately expressed with lactoferrin (Graubert *et al.*, 1993). It cleaves type IV basement membrane collagen into 1 of 4 amino terminal and 3 of 4 carboxyl terminal fragments andf is also able to cleave fibronectin, types V and VII collagen and denatured collagen (gelatin) (Morel *et al.*, 1993).

2.C.6.2. Neutrophil serine proteases

The serine proteases are a family of enzymes which utilise a uniquely activated serine residue in the substrate binding site to catalytically hydrolyse peptide bonds (Devlin, 1992). They are characteristically inhibited by diisopropyl fluorophosphate (DFP) and phenylmethylsulphonyl fluoride (PMSF) which interact with this serine residue within the active site of the enzyme (Stryer, 1981).

2.C.6.2.1. Human neutrophil elastase (HNE)

HNE has sparked quite an interest due to its putative role in the pathogenesis of pulmonary emphysema. HNE activity has also been implicated in the pathology of ARDS and rheumatoid arthritis (Thomas *et al.*, 1991). The specific substrate for elastase is elastin and it attacks the peptide bonds on the carboxyl side of valine and, to a lesser extent, alanine. HNE has a pI of 5.5-5.9, a molecular mass of 30 kDa and is specifically inhibited by the serpin α_1PI, which has recently been found to exist in the neutrophil cytosol (Thomas *et al.*, 1991). The cytosolic inhibition of HNE may represent a means of protecting the intracellular environment from proteolytic injury during degranulation. HNE-α_1PI complexes have been found to be chemotactic for neutrophils and an HNE-α_1PI receptor has been identified on the neutrophil surface which mediates endocytosis and catabolism of HNE-α_1PI complexes (Joslin *et al.*, 1992). Reduced levels of α_1PI in the plasma, which manifests as a disorder described as α_1PI deficiency, leads to unopposed protease activity in the lungs and culminates in pulmonary emphysema (Thomas *et al.*, 1991). HNE is susceptible to other inhibitors besides α_1PI, namely, the general serine protease inhibitors, α_2-macroglobulin, antileukoprotease (or secretory leukocyte protease inhibitor) as well as $(Ala)_2$-Pro-Val-chloromethyl ketone. Despite the definite specificity of HNE for elastin, HNE has been shown to have broad substrate specificity for many extracellular matrix proteins e.g. the endothelial proteoglycans (Key *et al.*, 1992), collagens, fibronectin, fbg, basement membrane and immunoglobulins (Thomas *et al.*, 1991). As mentioned above, a membrane form of HNE, which is the result of rebinding of secreted HNE to the neutrophil surface, has been noted (Bangalore and Travis, 1994). This membrane form of HNE demonstrates resistance to HNE inhibitors including α_1PI.

A study done by Woodman *et al.* (1993) demonstrated that aside from the destructive potential of HNE at inflammatory sites it also has the ability to upregulate the expression of the β subunit of the CD11/CD18 integrin complex and to a lesser extent CD11b on the neutrophil surface (see 2.C.7.3.1. Neutrophil integrins). This upregulation was found to affect neutrophil migration, but not adhesion, at inflammatory sites, although the mechanism for this remains unclear. HNE has also been implicated in the priming of macrophage superoxide production, modification of platelet aggregation and stimulation of B cell proliferation (Kusner and King, 1989).

2.C.6.2.2. Cathepsin G

Cathepsin G is a 29 kDa chymotrypsin-like serine protease which is also capable of cleaving elastin but in a manner distinct to HNE as it attacks bonds on the carboxyl side of tyrosine and phenylalanine. Like HNE, cathepsin G has a pI of 5.5-5.9 and is specifically inhibited by the cytosolic α_1PI (Kusner and King, 1989). It is released in significant quantities at sites of inflammation. The precise function of cathepsin G is ill defined but it has been suggested to function as an immune response modifier. Examples of this include its ability to a) prime macrophage superoxide response, b) enhance phagocytosis of immune complexes by neutrophils, c) cleave human IgG, IgM, fbg, fibronectin and C1, C4, C3 and C5 and d) stimulate B cell proliferation (from Kusner and King, 1989). Like HNE, secreted cathepsin G has been shown to partially rebind to the neutrophil surface and consequently demonstrates anti-protease resistant behaviour (Bangalore and Travis, 1994, see 2.B.7.2. Digestion of fbg by non-plasmin mediated pathways).

2.C.6.2.3. Proteinase 3 (PR3)

PR3 was first discovered in 1978 by Bagglioni *et al.* who showed its electrophoretic mobility to differ from that of HNE and cathepsin G (from Kao *et al.*, 1988). PR3 was initially found to be able to cause emphysema in hamsters with a severity equal to, if not greater, than that caused by HNE (Kao *et al.*, 1988). PR3 is able to degrade elastin more efficiently than HNE at pH 6.5 but not at pH 7.4 or 8.9. Besides being able to degrade elastin, PR3 has been shown to have broad substrate specificity, similar to that of HNE and cathepsin G, and may cleave fibronectin, laminin, vitronectin and collagen type IV but not interstitial collagens type I and III (Rao *et al.*, 1991). The substrate specificity of PR3 indicates a possible role in the migration of neutrophils through the endothelium. The pI of PR3 is less than HNE and cathepsin G and it is also immunologically distinct from HNE (Kao *et al.*, 1988). The exact sites of attack of PR3 on elastin were not determined but were suggested to be different from those of HNE and cathepsin G (Kao *et al.*, 1988) although Rao *et*

al. (1991) showed that PR3, like HNE, prefers small aliphatic amino acids (alanine, serine and valine). PR3 bears resemblance to myeloblastin and has been implicated in myeloblast differentiation. The cDNA of PR3 has been isolated and cloned and the predicted amino acid sequence was found to be highly homologous to the primary structure of elastase, cathepsin G and other serine proteases and it was suggested that the genes for these three proteins share a common progenitor (Campanelli *et al.*, 1990; Sturrock *et al.*, 1992). The PR3 gene is on human chromosome 19 which was thought to be unique for the trypsin-like serine proteases (Sturrock *et al.*, 1992) but work by Zimmer et al. (1992) showed PR3 to be a member of a cluster of elastase-related genes in the terminal region of the short arm of chromosome 19. The other two members are elastase and azurocidin (an antimicrobial substance of the primary granules, see below) and all three are co-ordinately expressed. PR3 was shown to share both the catalytic triad and the substrate binding pocket of elastase. There are a few small differences in the substrate binding pockets of the two proteases with one or two amino acid substitutions (Val-168 to Ile in PR3 and Ala-187 to Asp in PR3) and insertions in PR3 (between the Gly-22 and Gly-23 and between the Gly-23 and His-24 of HNE) which may explain the slight differences in reactivity of PR3 for the HNE substrates (Rao *et al.*, 1991). PR3 is inhibitable with PMSF, DFP, α_1PI and α_2-macroglobulin but not α_1-anti-chymotrypsin and secretory leukocyte protease inhibitor. PR3 is also inhibited by the elastase specific chloromethyl ketone inhibitor, MeO-Suc-Ala_2-Pro-Val-CH_2Cl, but less effectively than HNE, and is not inhibited at all by MeO-Suc-Ala_2-Pro-Ala-CH_2Cl (Rao *et al.*, 1991). PR3 was found to exist in isoenzyme forms with the predominant form being approximately 26 kDa and, similarly to elastase, it is localised in the neutrophil primary granules (Kao *et al.*, 1988).

PR3 may also be expressed, following stimulation with TNF-α and IL-8, in small amounts on the plasma membrane (Csernok *et al.*, 1990; Csernok *et al.*, 1994) and this correlates with release of the primary granule contents from the cell but is not associated with the translocation of MPO or elastase to the plasma membrane. This may suggest that PR3 has a different accessibility to the membrane when compared with other azurophil granule components, although the mechanism for this is not understood. Interestingly, PR3 is more abundant than HNE and may be responsible for functions previously attributed to HNE (Campanelli *et al.*, 1990). An important pathological finding is the ability of PR3 to act as an autoantigen in the generation of anti-neutrophil cytoplasmic antibodies (ANCA) in Wegeners granulomatosis (WG), which is a systemic necrotising vasculitis associated with granuloma formation (Csernok *et al.*, 1990; Csernok *et al.*, 1994).

2.C.6.2.4. Azurocidin and azurophil granule protein 7 (AGP7)

Azurocidin and AGP7 are both found in the primary granules of the neutrophil and show considerable sequence homology to elastase (Wilde *et al.*, 1990). Azurocidin has been shown to share 65% sequence homology with the active site of elastase. Despite this homology, the catalytic serine is replaced with a glycine in azurocidin and it is subsequently unable to bind DFP (Wilde *et al.*, 1990). Despite the lack of proteolytic activity, azurocidin has been demonstrated to display antimicrobial activity *in vitro*. AGP7, on the other hand, shows 70% homology with elastase at the active site and is capable of binding DFP, thus demonstrating active serine proteolytic activity. Azurocidin comprises three predominant bands on SDS-PAGE with molecular mass values 28-30 kDa and AGP7 comprises four distinct glycoforms of molecular mass values 28-34 kDa (Wilde *et al.*, 1990). Both these proteins, and in particular AGP7, are extremely insoluble at neutral pH and it was suggested that they may be associated with the neutrophil membrane. A relationship between PR3 and AGP7 has also been suggested and is under investigation (Wilde *et al.*, 1990).

2.C.6.3. Neutrophil membrane-associated proteases

A serine protease which differs in its catalytic and molecular properties to the well known proteases of the primary granules (see above) was isolated from the neutrophil membrane (Pontremoli *et al.*, 1986). This protease is released into the extracellular medium with stimulation of low concentrations of PMA (optimally at 10 ng/ml) and this release is concomitant with the production of oxygen radicals. Contribution of the secondary granules was excluded due to the observation of only limited release of the secondary granule constituents with low concentrations of PMA (Pontremoli *et al.*, 1986). The release of this protease from the membrane was found by Melloni *et al.* (1986) to be dependent on ATP and PKC activation. It was proposed to have a role in the cytolytic effects of the neutrophil, in co-operation with the oxygen radicals. The protease was characterised as a high molecular mass (approximately 300 kDa), Ca^{2+} independent enzyme with a pH optimum between 7.4-7.8 (Pontremoli *et al.*, 1986). Subsequent to the identification of this membrane associated neutral serine protease Pontremoli *et al.* (1990) noted that it is in fact localised at sites of interaction of the neutrophil cytoskeletal proteins with the cell membrane. They also noted an activity of this serine protease for the modification and down-regulation of the PKC isoforms following PMA or FMLP stimulation.

The existence of a chymotrypsin-like protease in the neutrophil membrane has been noted by King *et al.* (1986). This membrane protease was initially described with an approximate molecular mass of 150-180 kDa (King *et al.*, 1986) but was later shown to be approximately 65-70 kDa with an isoelectric point of 6.3 and displaying Ca^{2+} dependent serine protease activity (King *et al.*, 1991). The murine anti-human neutrophil IgG_1 monoclonal antibody, Ab 1-15, is specific for this enzyme and inhibits activity related to cell activation or more specifically O_2^- generation and cAMP production, following FMLP or PMA stimulation (King *et al.*, 1987). The mechanism of cellular activity modulation by this serine protease was not ascertained but was suggested to be due to the proteolytic alteration and activation of secondary messenger pathways situated in the neutrophil membrane. The protease was found in association with the FMLP receptor, which is known to mediate neutrophil activation by the stimulation of NADPH oxidase as well as a neutrophil-specific, guanosine-dependent regulatory G protein which activates phospholipase C. It was initially suggested that the membrane associated serine protease forms part of the FMLP receptor complex on the neutrophil. It was later proposed, however, that this membrane enzyme represents a low affinity FMLP binding protein distinct from the cell activating FMLP receptor which may also be involved in degradation of the FMLP receptor/ligand (King *et al.*, 1991). The exact role of this membrane bound protease was not identified.

Having demonstrated a membrane enzyme in the neutrophil which is associated with the initiation of the superoxide response in the presence of inflammatory stimuli (King *et al.*, 1987), a subsequent study by Kusner and King (1989) was of interest. They showed that local release of the neutrophil primary granule neutral proteases, cathepsin G and elastase, into the extracellular medium can also augment neutrophil effector function by upregulating oxidative responses to inflammatory stimuli.

A 100 kDa neutral endopeptidase (NEP) has also been found in the neutrophil plasma membrane which is capable of cleaving FMLP at the Met-Leu bond with subsequent inhibition of FMLP mediated chemotaxis (Connelly *et al.*, 1985; Painter *et al.*, 1988). This protease was identified as a metalloprotease which is constitutively expressed in the membrane and does not require neutrophil activation for expression. The neutrophil NEP was found to be identical to the kidney-brush border neutral proteinase and the brain enkephalinase. NEP was found to modulate the chemotactic response of neutrophils by reducing FMLP concentration suggesting a mechanism of controlling the amount of chemotactic stimuli interacting with the neutrophil in the face of overwhelming concentrations of the chemoattractant. Patients presenting with ARDS and especially when complicated with septic pneumonia, show abnormally high levels of this enzyme (Connelly *et al.*, 1985).

2.C.7. NEUTROPHIL ADHESION

The migration of neutrophils from the vasculature, through the endothelium and into the tissue, toward sites of inflammation, involves a complex series of events and a number of adhesion receptors on both the neutrophil and the endothelium (Zimmerman *et al.*, 1992; Bevilacqua, 1993; Adams and Shaw, 1994) (Fig 3). The sequence of events of neutrophil adhesion and migration has been documented as follows: initially the neutrophils appear to 'slow down' and 'roll' along the endothelial surface. This so-called 'rolling' of the neutrophils is mediated by the interaction of the selectins on both the neutrophil and endothelial surface and is the initiating process for subsequent 'tighter' binding and movement of the neutrophils through the endothelium, mediated by neutrophil integrins and receptors of the immunoglobulin superfamily on the endothelium (Fig 3). Neutrophil migration utilises a combination of regulated and reversible adherence processes to the matrix components which is a necessary requirement for the production of reactive oxygen species and release of degradative proteases (from Adams and Shaw, 1994).

In order to achieve a complete understanding of the adhesive process and functions of the neutrophil a brief outline of the selectins, the immunoglobulin superfamily and the integrins, which play a role in this process, will be provided.

2.C.7.1. The selectins

The selectins consist of a group of three glycoproteins which are expressed on the endothelium, platelets and leukocytes and interact with specific carbohydrates (Bevilacqua and Nelson, 1993). The nomenclature of the three selectins has been confusing in the past but are now named according to the cell type from which they were originally identified, namely, E-selectin (endothelium), P-selectin (platelets) and L-selectin (lymphocytes). The 'old' names of the selectins include endothelial-leukocyte adhesion molecule 1 (ELAM-1) (now E-selectin), platelet activation dependent granule-external membrane protein (PADGEM) or granule membrane protein 140 (GMP-140) or CD62 (now P-selectin) and murine lymph node homing receptor (mLHR) or MEL-14 antigen or leukocyte-endothelial cell adhesion molecule 1 (LECAM-1) (now L-selectin) (Bevilacqua and Nelson, 1993).

E-selectin expression is largely restricted to activated endothelial cells and supports the adhesion of neutrophils, monocytes and a subpopulation of memory T-lymphocytes. Endotoxin, IL-1 or TNF may stimulate synthesis and expression of E-selectin within 4-6 hr (Bevilacqua and Nelson, 1993).

P-selectin has a molecular mass of approximately 140 kDa and is associated with the α- and dense-granules in resting platelets and the Weibel-Palade bodies of inactive endothelium. Thrombin, histamine, terminal complement compounds and H_2O_2 stimulate the immediate redistribution of P-selectin to the platelet or endothelial surface where, in the endothelial cells, it is then reinternalised and its expression declines within minutes (Zimmerman *et al.*, 1992). P-selectin contains a sequence within its cytoplasmic domain which routes it for degradation in the lysosomes following internalisation. This constitutive mechanism for the down-regulation of P-selectin is unusual in that it does not require an extracellular signal (Green *et al.*, 1994). P-selectin expression on platelets and endothelium may support initial leukocyte adhesion (Bevilacqua and Nelson, 1993).

L-selectin is constitutively expressed on most circulating human lymphocytes, neutrophils and monocytes and supports their adhesion to endothelium (Bevilacqua and Nelson, 1993). Activation of the cells results in a change in avidity of L-selectin rather than its expression and downregulation of the receptor is achieved by the 'shedding' of L-selectin from the cell surface (Bevilacqua and Nelson, 1993). Pizcueta and Luskinskas (1994) have recently noted a role for L-selectin in the recruitment of neutrophils during chronic inflammation (up to 48 hr) in contrast with previous studies which have all looked at initial adhesive interactions (2-4 hr).

The selectins are typically Type I transmembrane proteins with a well conserved N-terminal lectin-like domain, an EGF repeat and a variable number of modules (approximately 60 amino acids each) similar to those found in certain complement binding proteins (Bevilacqua and Nelson, 1993). The lectin and EGF domains have been found to be crucial for selectin mediated adhesion. The function of the complement binding-like region is not known but is proposed to 'hold' the other two regions away from the cell surface thereby enhancing adhesive function. Selectins require Ca^{2+} for activity (Bevilacqua and Nelson, 1993).

The specific ligands for the selectins are carbohydrate containing compounds (Bevilacqua and Nelson, 1993). L-selectin binds to phosphorylated monosaccharides such as mannose-6-phosphate as well as certain sulphated polysaccharides such as fucoidan and sulphatides, namely, 3-0-

sulphate-galactosyl ceramide. P-selectin may also bind to fucoidin, heparin and 3-0-sulphate-galactosyl ceramide. More recently oligosaccharide structures related to the fucosylated lactosamines, namely, sialylated Lewis x (sLe^x, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc) and sialylated Lewis a (sLe^a, Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc) have been identified as important selectin ligands. sLe^x and other fucosylated lactosamines are found in abundance on circulating neutrophils and monocytes which would support their adhesion to the endothelium via E-selectin. sLe^a is not typically expressed on leukocytes but has been found on cancer cells and it is suggested to play a role in metastasis (Bevilacqua and Nelson, 1993). Of interest is the theory that L-selectin may represent a ligand for E-selectin on the endothelium (Bevilacqua and Nelson, 1993). P-selectin also appears to recognise its ligand when presented in a glycoprotein form but the nature of participation of protein and carbohydrate in these selectin ligands is not known (Bevilacqua and Nelson 1993).

An interesting feature of the selectins is the fact they have soluble isoforms which can be found in the circulation (Gearing and Newman, 1993). P-selectin was shown to have an alternately spliced mRNA lacking a transmembrane domain which shows only transient expression on the cell surface. L-selectin is cleaved from the cell surface in a proteolytic fashion and E-selectin appears to be lost from the cell surface within 24 hr of expression. These soluble isoforms are bioactive, can inhibit cellular adhesion and have been found to be raised in association with disease activity, for example, in patients with sepsis, HIV, haemolytic uremic syndrome, SLE, acute *Plasmodium falciparum* malaria and diabetes (Gearing and Newman, 1993). It is tenable that these circulating adhesion molecules may be useful monitors of disease activity.

2.C.7.2. The immunoglobulin superfamily-intercellular adhesion molecules (ICAM'S)

This family of adhesion molecules is characterised structurally by repeated immunoglobulin-like domains in the extracellular domain. The numerous members of the immunoglobulin superfamily (IgSF) are believed to have evolved from a single ancestral unit, the Ig fold (Garrod, 1993; Pigott and Power, 1993) which is typically 70-110 amino acids organised into two parallel β sheets and stabilised with disulphide bonds (Pigott and Power, 1993).

The IgSF is vast and this review covers, only briefly, one group of receptors within this family, namely the intercellular cell adhesion molecules (ICAM's). The ICAM's consist of three members, ICAM-1, ICAM-2 and ICAM-3 which have varying numbers of Ig-like domains. ICAM-1 and

ICAM-2 contain five and two Ig-like domains, respectively, (Fougerolles and Springer, 1993) and ICAM-3 consists of five immunoglobulin domains with 15 N-linked glocosylation sites (Vives, 1994). As their name suggests the ICAM's are found as cell surface molecules although, as in the case of the selectins, soluble isoforms are known to exist (Fougerolles and Springer, 1993). A specific feature of the ICAM's is their ability to recognise the β_2 integrin LFA-1 (see below) and they are believed to function, not only as adhesive receptors, but, in the case of ICAM-3, as signal transducers (Vives, 1994).

ICAM-1 (CD54) is basally expressed in significant amounts on monocytes and is inducible on thymocytes, dendritic cells, endothelial cells, fibroblasts, keratinocytes, chondrocytes and epithelial cells (Pigott and Power, 1993). Induction of ICAM-1 expression may occur with the action of IFN-γ, IL1β, TNF-α and LPS. ICAM-2 (CD102) is constitutively expressed on endothelial cells, subpopulations of lymphocytes, monocytes, splenic sinusoids and dendritic cells. The functions of these receptors are numerous but of interest here is their ability to mediate the attachment of neutrophils to the endothelium. ICAM-3 (CD50) is constitutively and strongly expressed on resting lymphocytes and neutrophils (Vives, 1994; Fougerolles and Springer, 1993). Modulation of ICAM-3 expression on human neutrophils appears to involve a PMA-activated proteolytic shedding mechanism with the release of soluble ICAM-3 into the circulation (del Pozo *et al.*, 1994). The role of ICAM-3 interaction with LFA-1 on neutophil homotypic aggregation has not been investigated.

2.C.7.3. The integrins

The integrins are a family of membrane glycoproteins which exist as heterodimers with an α and β subunit (Fig 4). The α subunits vary in size between 120 and 180 kDa and are non-covalently associated with a β subunit which ranges from 90-110 kDa. Most cells express integrins and so far there are 8 known β subunits and 15 known α subunits (Smyth *et al.*, 1993). The integrin family is subdivided into classes based on the β subunit. The most widely distributed integrins belong to the β_1 class, also known as the very late antigens (VLA). The second class constitutes the leukocyte specific receptors or β_2 integrins and the third class are the β_3 integrins, otherwise described as the cytoadhesins. Less is known of the β_4 through to β_8 integrins (Smyth *et al.*, 1993). The integrins expressed by different cell types varies greatly although some integrins are definitely cell-type specific. For example, megakaryocytes and platelets exclusively express gpIIb/IIIa (β_3) while the β_2 integrins are present only on leukocytes (Ruoslahti, 1991).

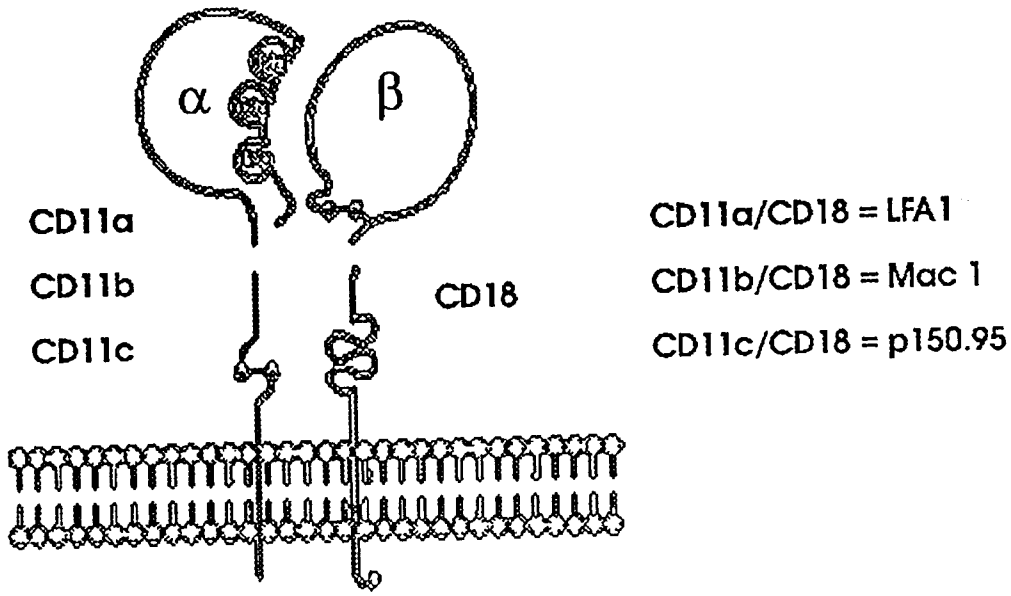

Figure 4. Schematic representation of a typical integrin. Integrins are a family of heterodimeric, transmembrane glycoproteins. The leukocyte specific or β_2 integrins possess a common β subunit (CD18) which may associate with one of three α subunits (CD11a, CD11b or CD11c) to give rise to CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1) and CD11c/CD18 (p150,95) (adapted from Pigott and Power, 1993).

A wide variety of proteins serve as ligands for integrin receptors and in general may be classified into three groups, namely, extracellular matrix proteins (collagen, fibronectin, fbg, laminin, thrombospondin and vitronectin), plasma proteins (fbg and factor X) and cell surface molecules (C3bi, ICAM-1,2,3 and VCAM-1) (Smyth *et al.*, 1993). Individual integrins can often bind to more than one ligand and similarly individual ligands may be recognised by more than one integrin. The tripeptide sequence R-G-D which is present in a number of ligands, e.g. fbg, fibronectin, thrombospondin, type 1 collagen and vitronectin, has been found to be a common recognition sequence for a number of integrins, particularly $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_4$ (Smyth *et al.*, 1993). The conformation of the R-G-D sequence appears to be critical for optimal binding as well as determining to which integrin it will bind (Ruoslahti, 1991; Gurrath *et al.*, 1992). Other ligand sequences include K-Q-A-G-D-V in fbg which is recognised by $\alpha_{IIb}\beta_3$ as well as the novel LRI on neutrophils (see below) (Gresham *et al.*, 1992). Asp-Gly-Glu-Ala (D-G-E-A) in collagen is recognised by $\alpha_2\beta_1$, Glu-Ile-Leu-Asp-Val (E-I-L-D-V) in fibronectin is recognised by $\alpha_4\beta_1$ and G-P-R-P in fbg is recognised by $\alpha_X\beta_2$. Synthetic peptides of these recognition sequences may mimic the activity of the intact protein but do not always bind with the same affinity as the native

protein indicating that conformation, or additional contact sites, may be important for optimal binding (Hynes, 1992; Smyth *et al.*, 1993). Another interesting feature of integrins is the expression of different ligand specificities by the same integrin on different cell types which is related to either cell-type specific structural modifications of the integrins or association with cell-type specific protein or lipid factors (Smyth *et al.*, 1993).

Both the α and the β subunit contain a single hydrophobic transmembrane segment with a short cytoplasmic carboxyl domain (with the exception of β_4) and a large extracellular amino terminal domain (Fig 4). The extracellular domains of the α and the β subunit associate with each other toward the globular, amino terminus to form the ligand binding pocket of the αβ subunit (Smyth *et al.*, 1993). It has been suggested that high affinity ligand recognition requires both α and β subunit participation (from Loftus *et al.*, 1994). The integrin cytoplasmic domains are believed to interact indirectly with the cytoskeletal filaments by associating with intermediary linker proteins e.g. talin and α-actinin (Pavalko and Otey, 1994). This association may result in the translation of the extracellular signal, following ligand binding, into an intracellular event by reorganisation of the cytoskeleton. The amino terminus is tightly folded with disulphide loops and contributes to the ligand binding domain (Hynes, 1992). Some α subunits contain an extra segment of about 180 amino acids called the I domain which is inserted directly before the cation binding domain. The exact function of the I domain is not really known but appears to contribute to ligand binding and recognition. (Diamond *et al.*, 1993; Hynes, 1992).

An interesting feature of integrins is that individual cells can vary their adhesive properties by selective expression of integrins as well as the ability to modulate the binding properties of the integrins. For example, the β_2 integrins on leukocytes are inactive and will not bind their respective ligands until the receptor is activated and a conformational change has taken place (Andrew *et al.*, 1993). This activation may be achieved with phorbol esters and various inflammatory mediators such as TNF, C5a, PAF or FMLP and may be crucial for localising the adhesion of leukocytes to inflammatory sites. The adherence of the leukocyte to the extracellular matrix via the β_2 integrins may itself lead to activation of the cell manifested in the induction of the respiratory burst, cell motility and Ca^{2+} transients in the cytoplasm (Hynes, 1992). It has been suggested that the interaction of the integrin with ligand may result in the exposure of novel binding sites described as 'ligand-induced binding sites' and subsequent increased binding affinity (Hogg *et al.*, 1993). Upregulation of the expression of integrins on various cells may also be influenced by various

growth and differentiation factors, namely, TNF- β, IFN-γ and retinoic acid (Smyth *et al.*, 1993). The avidity state of the integrin is transient and the ligand may 'de-adhere' following a period of time (Hogg *et al.*, 1993). The exact mechanism for this is not understood but clearly represents an important means for controlling integrin/ligand induced activity at inflammatory sites.

2.C.7.3.1. Neutrophil integrins

The β_2 integrins have been described as leukocyte specific and consist of $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (Mac-1) and $\alpha_X\beta_2$ (p150,95) (Fig 4). These are more commonly referred to using the CD nomenclature where the β subunit (CD18) is common and may associate with one of three α subunits resulting in CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1) and CD11c/CD18 (p150,95) (Hynes, 1992). β_2 integrins exist in both resting and active conformations and activation occurs rapidly and reversibly in response to soluble stimuli (see above). These stimuli increase intracellular Ca^{2+} concentrations, through kinase stimulation, resulting in phosphorylation of the β subunit. The cytoplasmic domain of CD18 also appears to associate with α-actinin in the cytoskeletal actin filaments, following cellular activation (Pavalko and LaRoche, 1993) (see above). Although these two processes of kinase activation and cytoskeletal rearrangement are known to occur in association with cellular activation following integrin/ligand interactions, the exact mechanism remains unknown. Strong evidence has been provided that phospholipase A_2 (PLA_2) activity may play a direct role in the regulation of expression and function of the Mac-1 integrin (Jacobson and Schrier, 1993). Of interest is the presence, in activated neutrophils only, of a lipid factor whose function appears to be the enhancement of ligand binding to the β_2 integrins CR3 and LFA-1 and has been described as integrin modulating factor-1 (IMF-1). It was suggested that neutrophils control adhesivity by controlling synthesis of IMF-1, which acts as an allosteric activator of neutrophil integrins. Degradation of IMF-1 may then lead to a relaxation of the β_2 integrin to a low avidity state. IMF-1 does not appear to affect the function of the β_1 or the β_3 integrins (Hermanowski-Vosatka *et al.*, 1992).

A unique leukocyte integrin was identified by Gresham *et al.* (1989) and isolated by Carreno *et al.* (1993). It was found to be distinct from, but most immunologically closely related to, the β3 integrins of the cytoadhesin family and was described as leukocyte response integrin (LRI). It typically binds to the basement membrane protein entactin but may also bind a number of other ligands including fbg. This receptor, when not activated, was shown to have ligand specificity for

the R-G-D sequence but following FMLP stimulation was shown to have a greater affinity for the K-Q-A-G-D-V sequence present in the carboxyl terminus of the γ chain of fbg and surprisingly showed the greatest affinity for a K-G-A-D-G-V peptide (Gresham *et al.*, 1992). The ability of LRI to interact with its ligand (via R-G-D) without prior activation, as well as the ability to bind the synthetic K-G-A-G-D-V peptide, distinguishes it from all other integrins. Another interesting feature of LRI is its physical and functional association with a separate 50 kDa protein described as integrin-associated protein (IAP), forming a signal transduction unit for the activation of phagocytosis (Brown *et al.*, 1990; Zhou and Brown, 1993). Binding of monoclonal antibody to LRI results in stimulation of IgG mediated phagocytosis and the K-Q-A-G-D-V peptide as well as monoclonal antibody to IAP are able to inhibit fbg stimulated phagocytosis by leukocytes (Gresham *et al.*, 1989; Gresham *et al.*, 1992). The binding of the R-G-D sequence in entactin to LRI has also been shown to elicit neutrophil adhesion and chemotaxis (Senior *et al.*, 1992) indicating an important role for this novel receptor in neutrophil modulation at inflammatory sites. LRI appears to exert its function early in the inflammatory response as it does not require activation for R-G-D-dependent ligand binding and it is inactivated by the products of the respiratory burst. It is, therefore, interesting to note that the LRI-IAP signal transduction unit itself is able to activate the phagocytic respiratory burst in a CD18-independent cell adhesion manner (Zhou and Brown, 1993).

2.C.7.4. Endothelial-Neutrophil interaction

Neutrophils act as the first line of defence against invading pathogens and acute tissue injury and it is thus imperative that they are able to rapidly migrate toward the site of inflammation. Of equal importance is that the number of neutrophils, the intensity and the duration of their action be controlled so as to prevent leukocyte mediated tissue damage. The neutrophils are able to exhibit adhesion and de-adhesion in a finely regulated manner and the inflammatory mediators which control this process are differentially expressed and regulated for this purpose (Zimmerman *et al.*, 1992).

Migration of neutrophils from the vasculature toward sites of inflammation takes place as a three step process. Neutrophils initially interact via the selectins which cause the cells to 'roll' along the endothelial surface (Fig 3). It is generally believed that E-selectin and P-selectin, on the endothelium, interact with L-selectin and surface glycoproteins containing fucosylated lactosamines on the neutrophil to allow for neutrophil 'rolling' along the endothelium (Bevilaqua, 1993). Ley *et al.* (1993) have, however, indicated that L-selectin alone can mediate leukocyte rolling under

conditions of shear stress. They also showed that L-selectin does not bind to E- or P-selectin but they could not identify the relevant endothelial ligand. Thrombin and histamine stimulate the redistribution of P-selectin from the endothelial storage granules within minutes whereas endotoxin, IL-1 or TNF may stimulate biosynthesis of E-selectin over a period of hours to days (Bevilacqua, 1993). 'Rolling' of the neutrophils or tethering of the neutrophil to endothelial P-selectin appears to be necessary for the neutrophil to 'sample' the endothelium for trigger factors (PAF and IL-8) necessary for optimal upregulation of integrin function and subsequent 'firm' attachment of the neutrophil to the endothelium (Zimmerman *et al.*, 1992; Adams and Shaw, 1994) (Fig 3). PAF and IL-8 are released from the endothelium in response to inflammatory stimuli (Zimmerman *et al.*, 1992; Adams and Shaw, 1994). Action of the selectins in 'slowing-down' of the neutrophils may also be important for optimal avidity to be achieved by the integrins, which are believed not to function under conditions of shear stress (Lawrence *et al.*, 1990; Zimmerman *et al.*, 1992).

The β_2 integrin, on the neutrophil, interacts with the immunoglobulin-related cell adhesion molecules (IgCAMs) on the endothelium, namely, ICAM-1 and ICAM-2 (Bevilacqua, 1993) (Fig 3). CD11b/CD18 adheres to ICAM-1 only whereas CD11a/CD18 recognises ICAM-1 and ICAM-2 (Zimmerman *et al.*, 1992). ICAM-1 is found in low levels on unstimulated endothelial cells and synthesis is induced by IL-1 and TNF over several hours. ICAM-2, on the other hand, is constitutively expressed and provides a ready surface for β_2 integrin adhesion. Activation of the neutrophil by activated endothelial associated PAF and IL-8, results in increased avidity of CD11a/CD18 and increases in number of CD11b/CD18 and CD11c/CD18 (Bevilacqua, 1993). Rochon and Frojmovic (1992) believe that activation of the endothelial cells alone is sufficient to allow adhesion of unactivated neutrophils. Once adhered, neutrophils are activated and serve to 'capture' other neutrophils, by means of their ability to aggregate and adhere to each other, thus overcoming shear stresses.

Following adherence neutrophils undergo a change of shape where they 'flatten' onto the endothelial surface and migrate into the underlying tissue toward the inflammatory site (Fig 3). This process of transendothelial migration is less understood but Vaporciyan *et al.* (1993) demonstrated that PECAM-1 (platelet-endothelial cell adhesion molecule 1 or CD31 of the Immunoglobulin Superfamily), expressed on both the neutrophils and endothelium, may play a role.

The importance of these interactions between endothelium and leukocyte is indicated in patients with leukocyte adhesion deficiency (LAD) who have reduced numbers of the β2 integrins and present with recurrent infections and an absence of neutrophils at sites of infection. This deficiency may often be fatal (from Adams and Shaw, 1994). However, besides host defence, leukocyte/endothelial interactions may also lead to the generation of unwanted pathological inflammation and consequently a thorough knowledge of these interactions could have clinical implications. Examples may include asthma, where antibodies to E-selectin may prevent antigen induced airway hyperresponsiveness by inhibiting neutrophil infiltration. Also, antibodies to ICAM-1 have shown promise in the prevention of graft rejection and treatment of rheumatoid arthritis (from Adams and Shaw, 1994).

2.C.7.5. Neutrophil-Extracellular Matrix interaction

The interaction of the neutrophil with components of the extracellular matrix (ECM), via the integrin receptors, may have important consequences for both ligand and cell in the inflammatory process (Juliano and Haskill, 1993). The translation of receptor occupancy into intracellular events (chemotaxis, phagocytosis, respiratory burst) is not fully understood and there are two, not necessarily mutually exclusive, views in this regard. Integrins have firstly been suggested to transmit signals by conformational changes associated with reorganisation of cytoskeletal components, thus regulating internal cellular organisation (Heidemann, 1993) and secondly, as a result of tyrosine phosphorylation and Ca^{2+} channel flux (Smyth *et al.*, 1993; Juliano and Haskill, 1993). Heidemann (1993) has demonstrated that integrins colocalise with the actin bundles and actin binding proteins in the cytoskeleton and can bind, *in vitro*, to talin and α-actinin. The different integrin α subunits may trigger different intracellular functions and different ligands for the same integrin may also elicit different intracellular responses (Juliano and Haskill, 1993).

Neutrophils are able to interact with a number of ECM components, namely fbg, laminin, fibronectin, gelatin, collagen IV and vitronectin (Singer *et al.*, 1989; Brown and Goodwin, 1988; Bohnsack *et al.*, 1990). The adherence of activated neutrophils to fibronectin has been shown to result in complement receptor mediated phagocytosis (Brown and Goodwin, 1988). An interesting feature of the adherence of neutrophils to laminin is the fact that, although it is predominantly β_2 integrin mediated, adherence also appears to occur via a β_1 VLA-like integrin (Bohnsack *et al.*, 1990).

In the case of fbg interaction with neutrophils the specific stimulator or agonist determines the specific neutrophil receptor which interacts with fbg. Phorbol esters stimulate adhesion of neutrophils to fbg via CD11b/CD18 while TNF stimulated neutrophils adhere via the CD11c/CD18 receptor (Wright *et al.*, 1988; Loike *et al.*, 1991). Neutrophils stimulated with TNF adhere to fbg coated surfaces via the CD11c/CD18 (p150,95) integrin (Loike *et al.*, 1991). Fbg is also able to bind to the neutrophil via the LRI (see above). Most integrins, including the β_2 integrin, recognise their ligands via the specific tri-peptide sequence, R-G-D (Smyth *et al.*, 1993). Fbg, on the other hand, interacts with the CD11/CD18 integrin via a unique structural motif. CD11c/CD18 interacts with fbg via the G-P-R sequence at positions 17-19 of the amino terminus of the Aα chain (Loike *et al.*, 1991) and CD11b/CD18 and LRI interact with the K-Q-A-G-D-V sequence at the carboxyl terminus of the γ chain (Wright *et al.*, 1988; Gresham *et al.*, 1992). Altieri *et al.* (1993) showed that Mac-1, on chemoattractant (FMLP) stimulated neutrophils and monocytes, interacts with a 30 kDa plasmic fragment D of fbg which lacks the R-G-D sequence as well as the carboxyl terminus of the γ chain. The binding of this fragment was found to occur via the Gly-190 to Val-202 sequence of the γ chain, shown by the ability of this sequence to block adhesion of these neutrophils and monocytes to immobilised fbg as well as to block adhesion of fbg to isolated CD11b/CD18. This sequence was also able to support adhesion of monocytes in a CD11b/CD18 dependent manner. Diamond and Springer (1993) demonstrated that binding of neutrophils to fbg occurs via a small subpopulation (10%) of the total CD11b/CD18 present on the neutrophil.

Studies have shown that the I-domain on the α chain of Mac-1 represents the recognition site for fbg. The amino terminus of the α chain and a divalent cation binding region also co-operatively contribute to ligand binding specificity despite being separated by the 200 amino acids of the I domain (Diamond *et al.*, 1993).

CHAPTER 3

MATERIALS AND METHODS

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REAGENTS

Monoclonal antibodies (all IgG1) to CD16 (FcRIII, low avidity Fc receptor), CD11a (LFA-1 α chain), CD11b (Mac-1 α chain), CD11c (p150,95 α chain), CD18 (CR3 β chain), and CD41a (intact gpIIb/IIIa, β_3 integrin family) were obtained from Sanbio, Uden, Netherlands. The monoclonal antibodies OKM1 (IgG2b) and OKM10 (IgG2a) (both directed against CD11b) were obtained from Ortho Pharmaceuticals, Raritan, NJ. Monoclonal antibodies to CD11a (IgG2a), CD11c (IgG1) and CD18 (IgG1) were also purchased from Serotec, Oxford, England. 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF, Pefabloc® SC) (serine protease inhibitor) and bisindolylmaleimide (protein kinase C [PKC] inhibitor) were obtained from Boehringer Mannheim, Germany. [^{125}I]-Na and $Na_2[^{51}Cr]O_3$ was from Amersham International, England. A kit containing prestained molecular weight markers for use in sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was obtained from Amersham International, Amersham, UK. The kit includes myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21 kDa) and lysozyme (14 kDa). Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY). A Sephacryl S-300 and S-200 gel filtration matrix as well as a Sephadex G25 matrix were obtained from Pharmacia, Uppsala, Sweden. All other chemicals and reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

3.1. PREPARATION AND IODINATION OF CRP

A pool of acute phase sera was prepared from patients 48 hrs post-surgery. Informed consent and ethical committee approval was obtained. CRP was isolated from the acute phase sera using calcium-dependent phosphorylethanolamine affinity chromatography and Sephacryl S-200 gel filtration (De Beer and Pepys, 1982). The pure CRP migrated as a single homogenous band with an apparent molecular mass of 24 kDa when subjected to 12% SDS-PAGE (reduced) (Laemmli, 1970). The CRP was iodinated using 0.5 μCi [^{125}I]-Na/μg CRP and Iodogen (Pierce Warriner, Cheshire, England) as an oxidising agent (Fraker and Speck, 1978). Iodine, not incorporated into the protein, was separated from the protein-bound iodine on a Sephadex G25 column. [^{125}I]-CRP maintained its binding capacity to CPS and >98% of the radioactivity associated with the CRP was precipitable by trichloracetic acid (TCA, 10% w/v). The specific activity of the [^{125}I]-labelled CRP was found to be 0.4 μCi/μg protein. The purity of the iodinated CRP was confirmed by 12% SDS-PAGE (reduced) (Laemmli, 1970) and autoradiography (400 000 cpm/track).

3.2. PREPARATION AND IODINATION OF FIBRINOGEN

Fbg was isolated by ammonium sulphate precipitation of plasma obtained from heparanised blood donated from healthy laboratory workers (Kalvaria *et al.*, 1986). The pure fbg migrated on 5-20% SDS-PAGE (non-reduced) (Laemmli, 1970) as a single band corresponding to the apparent molecular mass of 340 kDa. This preparation contained a trace amount of factor XIII which was used to catalyse crosslinking during fibrin formation. The clottability of the purified fbg was 97%. Fbg was iodinated using Iodogen (Pierce and Warner, Cheshire, England) as an oxidising agent and 0.5 μCi [^{125}I]-Na/μg fbg (Amersham International, England) (Fraker and Speck, 1978). The specific activity of the [^{125}I]-labelled fbg was 0.5 μCi/μg protein. SDS-PAGE (5-20% non-reduced) (Laemmli, 1970) and autoradiograph analysis of this [^{125}I]-labelled fbg (400 000cpm/track) revealed no evidence of degradation.

3.3. PREPARATION OF NEUTROPHILS, NEUTROPHIL MEMBRANES, CYTOSKELETON, CONDITIONED MEDIUM AND NEUTROPHIL LYSOSOMAL ENZYMES

Neutrophils were isolated from heparinised blood (5U preservative-free heparin/ml blood), donated by healthy laboratory workers, by hypaque/ficoll gradient centrifugation followed by hypotonic lysis of the red blood cells (Boyum, 1968). The neutrophils were 97-99% viable as determined by the exclusion of trypan blue and were found to be 98-100% polymorphonuclear using differential counts made on Wright-Giemsa stained smears. Neutrophils were resuspended either in phosphate-buffered saline (PBS) or in hanks balanced salt solution (HBSS) (Highveld Biological, JHB). The neutrophils were used immediately following purification.

Neutrophil conditioned medium was prepared by incubating neutrophils in the absence or presence of phorbol 12-myristate 13-acetate (PMA, 5ng/ml for fbg degradation and 10 ng/ml for CRP degradation) at 37^{o}C for 20 min (Shephard *et al.*, 1989). These doses of PMA and period of incubation released insignificant amounts of β-glucuronidase and a maximum of 12% of the total vitamin B_{12}-binding protein of the intact cells (Shephard *et al.*, 1989; Pontremoli *et al.*, 1986). Centrifugation at 400 g for 5 min at 4^{o}C removed the cells and the cell-free neutrophil conditioned medium was used within 5 min of collection. When the conditioned medium was to be loaded on a Sephacryl S-300 chromatography column (see below) it was concentrated to 1 ml on an Amicon PM-10 membrane.

Neutrophil cytoskeletons were isolated by lysing neutrophils ($40x10^6$/ml) for 10 min on ice in lysis buffer (50mM Tris-HCl, pH 7.5, containing 160 mM KCl, 10 mM EDTA and 1% (v/v) Triton X-100) (Yassim *et al.*, 1985). The triton insoluble cytoskeletons were pelleted by centrifuging at 12 000 g for 10 min at 4°C, then washed in the lysis buffer without Triton X-100.

Neutrophil membranes were prepared by lysing the cells ($25x10^6$/ml) in a hypotonic lysis buffer (20 mM Tris [pH 9.6], 10 mM NaCl, 5 mM $MgCl_2$ and 1mM ATP) for 20 min at 4°C (Shephard *et al.*, 1989). The nuclei were removed by centrifugation at 400 g for 5 min followed by centrifugation of the supernatant at 10 000 g for 1 hr, to yield a membrane-rich pellet. The pellet was resuspended in 7% (w/w) sucrose in 10 mM Tris (pH 7.5) and 5 mM EDTA (Tris-EDTA) and layered onto a stepwise sucrose gradient in this buffer (50% [w/w, 5 ml], 35% [w/w, 10 ml], 20% [w/w, 10 ml]) and centrifuged at 80 000 g for 3 hr (Beckman, SW 27 rotor). The membrane fraction was collected at the 20%/35% interface, washed three times with Tris-EDTA and finally resuspended in 10 mM Tris (pH 7.4) and 140 mM NaCL (Tris-NaCl).

The neutrophil cytoskeleton and membrane proteins were solubilised in 10 mM Tris-HCl (pH 7.4) containing 1M NaCl, for 1 hr on ice. Insoluble protein was removed by microfuging at 12 000 g for 5 min at 4°C. The solubilised proteins were dialysed through a Spectrapor membrane, molecular mass cut off 3.5 kDa (Spectrum Medical Industries Inc.,California), against PBS (pH 7.4, 4°C) and protein content was determined by the method of Lowry *et al.* (1951) using BSA as a standard. The membrane fraction was identified by the plasma membrane marker alkaline phosphatase and was found to be devoid of lysozyme, β-glucuronidase and vitamin B_{12}-binding protein (Shephard *et al.*, 1989).

Neutrophil lysosomal enzymes were obtained by degranulating neutrophils ($5x10^6$) with the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM) for 15 min at 37°C in the presence of cytochalasin B (5 μg/ml) (Bentwood and Henson, 1980). Centrifugation at 300 g for 5 min at 4°C removed the cells and the lysosomal-rich supernatant was used immediately after preparation.

3.4. INTERACTION OF CRP WITH NEUTROPHILS

3.4.1. Association of [^{125}I]-labelled CRP with neutrophils and concomitant degradation

[^{125}I]-labelled CRP (0-100 μg) and PMA (10 ng/ml) were incubated with neutrophils ($10x10^6$) prewarmed to 37 °C (15 min), in a final volume of 1 ml PBS and in the absence or presence of AEBSF (1 mM). When the effect of $CaCl_2$ on the association of CRP with neutrophils was investigated the incubation buffer was a 0.5 mM Na_2HPO_4 buffer (containing 1.2 mM $CaCl_2$, 140 mM NaCl, 4 mM KCl, 5 mM D-glucose, 20 mM HEPES and 1 mM $MgCl_2$, pH 7.4). In monoclonal antibody inhibition experiments PMA-stimulated neutrophils ($10x10^6$) were also preincubated (37°C, 15 min) without or with AEBSF (1 mM) with saturating concentrations (as determined by flow cytometry) of monoclonal antibodies directed against CD11c (Serotec), CD11a (Sanbio 1020), CD11b (Sanbio 1019), CD18 (Sanbio 1110), CD16 (Sanbio 1041c) and CD41a (Sanbio 1144 and Sanbio 1145 directed against Fc dependent reactions and fbg binding sites on platelets respectively) followed by the addition of the [^{125}I]-labelled CRP. All reactions looking at association of CRP with neutrophils were set up in duplicate and were terminated at specified time points (see results) by washing the cells three times with the incubation buffer at 4°C followed by transfer of the cells to clean tubes. The total radioactivity associated with the cells was quantified by gamma counting. The duplicates did not differ by more than 10% from each other and the mean of the duplicates was calculated. Non-specific binding was determined in the presence of both a 250 fold and 50 fold molar excess of unlabelled CRP. The nature of the neutrophil associated [^{125}I]-labelled CRP, in the presence of AEBSF, with and without a 250 fold or 50 fold molar excess of unlabelled CRP, was assessed by solubilising the final pellet of washed neutrophils with 100 μl of 20 mM Tris (pH 8.3), 1% (w/v) sodium decyl sulphate, and 7 M Urea followed by the addition of 50 μl SDS-sample buffer (0.012 M Tris [pH 6.8] with 4% SDS and 24% glycerol) and analysing on SDS-PAGE (5-20%) (Laemmli, 1970). The gels were dried immediately and autoradiographed. Degradation of the [^{125}I]-labelled CRP, during the course of the association of [^{125}I]-labelled CRP with neutrophils was monitored by 10% (w/v) TCA-soluble peptide formation (Shephard *et al.*, 1989).

3.4.2. Degradation of [^{125}I]-labelled CRP by neutrophil conditioned medium, neutrophil membranes, neutrophil cytoskeleton and neutrophil lysosomal enzymes

CRP degradation reactions contained 100 µg [^{125}I]-labelled CRP and the enzyme source in 1 ml PBS without $CaCl_2$ and $MgCl_2$. The enzyme source was either neutrophil conditioned medium from $10x10^6$ non-stimulated or PMA (10 ng/ml)-stimulated neutrophils ($10x10^6$), neutrophil membranes (100 µg), neutrophil cytoskeleton (100 µg) or lysosomal enzymes from $5x10^6$ neutrophils. When required, inhibitors, as stated in the results, were preincubated (37°C, 15 min) with the enzyme source before the addition of [^{125}I]-labelled CRP. [^{125}I]-labelled CRP degradation was monitored qualitatively at specific times by mixing aliquots of the reaction with an equal volume of SDS-sample buffer (0.012 M Tris [pH 6.8] with 4% (w/v) SDS, 24% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol) and analysing by SDS-PAGE (12%) (Laemmli, 1970) followed by autoradiography (Shephard *et al.*, 1989). Degradation was measured quantitatively by 10% TCA-soluble peptide formation. A 50 µl aliquot of the reaction mixture was precipitated with 50% TCA (w/v) (4°C) to give a final concentration of 10% (w/v) (Shephard *et al.*, 1989).

3.4.3. Influence of CRP on the release of CRP-degrading activity from neutrophils

Several studies were conducted to confirm whether CRP caused the release of proteolytic activity from neutrophils.

3.4.3.1. CRP-mediated release of proteolytic activity from neutrophils

To investigate the possibility that during an incubation of CRP with neutrophils, proteolytic activity is released from the cells into the extracellular medium, [^{125}I]-labelled CRP (100 µg) was incubated with non-stimulated and PMA-(10 ng/ml) stimulated neutrophils ($10x10^6$) for specific time periods (see results). The cells were then removed by centrifugation (400 g for 3 min at 4°C) and the extracellular medium returned to 37°C. Further degradation of [^{125}I]-labelled CRP in the cell-free extracellular medium was monitored as described above and compared with that had the cells not been removed from the reaction and that by neutrophil conditioned medium.

3.4.3.2. Quantification of the proteolytic activity released from neutrophils during an incubation with CRP

The proteolytic activity released by the neutrophil, in the presence of CRP, was quantified at the end point of CRP degradation. In order to do this a CRP-released conditioned medium was prepared by incubating unlabelled CRP (100 μg) with PMA-stimulated neutrophils ($10x10^6$) for 40 min, after which the cells were removed by centrifugation (400 g for 3 min) and the extracellular medium returned to 37°C for 20 hr. At this time point more than 90% of the unlabelled CRP in the extracellular medium had been degraded to TCA (10%, w/v)-soluble products and [^{125}I]-labelled CRP (100 μg) was then added. The conditions required for the generation of greater than 90% TCA-soluble peptides were determined in a duplicate reaction containing [^{125}I]-labelled CRP. Degradation of the [^{125}I]-labelled CRP by proteolytic activity released from neutrophils by unlabelled CRP, was quantified at various times as TCA soluble (10%, w/v) [^{125}I]-labelled peptide formation. This degradation was compared to that by PMA-stimulated neutrophils as well as a control conditioned medium which was subject to all the preparation steps described above, but in the absence of unlabelled CRP.

3.4.3.3. Quantification of CRP-degrading activity of the membrane and cytoskeleton of neutrophils following an incubation with CRP

Non-stimulated and PMA (10 ng/ml)-stimulated neutrophils ($10x10^6$) were incubated (37°C) with unlabelled CRP (2 μg or 500 μg) for 40 min whereupon the membrane and cytoskeleton fractions of the neutrophils were isolated (see above). These neutrophil fractions were incubated with [^{125}I]-labelled CRP (100 μg) and the generation of TCA (10%, w/v)-soluble peptides was compared to that of neutrophil cytoskeleton and membrane fractions isolated from control cells which had not been preincubated with unlabelled CRP.

3.4.4. Determination of the molecular size of the neutrophil CRP-degrading protease

3.4.4.1. SDS-PAGE analysis

Neutrophil membranes (200 μg), cytoskeletons (200 μg) and lyophilised conditioned medium from $100x10^6$ PMA stimulated neutrophils (prepared as described above) were analysed by 3-13% SDS-PAGE under non-reducing conditions (Laemmli, 1970). In order to recover enzyme activity the gels were washed for 45 min at 4°C in PBS/H_20, 1:1 (v/v) containing 0.2% (v/v) Triton X-100 followed by an additional 45 min wash in PBS/H_20, 1:1 (v/v) to remove the Triton X-100. The gels

were cut into 2 mm slices and each slice was assayed for activity by incubating it at 37°C with [^{125}I]-labelled CRP (100 μg) in a final volume of 1 ml PBS. TCA (10%, w/v)-soluble [^{125}I]-labelled CRP degradation products were measured (see 3.4.2.) at 18 hr. In order to visualise the protein within these three neutrophil fractions they were analysed on 3-13% SDS-PAGE (reduced and non-reduced) (Laemmli, 1970) followed by staining of the protein with 0.12% (w/v) coomassie blue. The three neutrophil fractions were also labelled with [^{125}I]-Na (150 μCi) (as described for the iodination of CRP in 3.1) and analysed by 3-13% SDS-PAGE (Laemmli, 1970) followed by autoradiography. A kit containing pre-stained protein molecular weight markers for molecular weights 14-200 kDa, ferritin (subunit size 220 kDa), purified human fbg (340 kDa) and fibronectin (440 kDa) were used as calibration standards.

3.4.4.2. Size Exclusion Chromatography

A Sephacryl S-300 column (1x45 cm) equilibrated with PBS, was loaded with either neutrophil membranes (500 μg), cytoskeletons (500 μg) or conditioned medium from 100x10^6 PMA-stimulated neutrophils. Fractions of 0.6 ml, at a flow rate of 3 ml/hr, were collected and assayed for CRP-degrading activity (see 3.4.2.). The peak fractions possessing proteolytic activity were iodinated and analysed by 3-13% SDS-PAGE (Laemmli, 1970) and autoradiography.

3.5. CHARACTERISATION OF FIBRINOGEN PROTEOLYSIS BY NEUTROPHILS

3.5.1 Degradation of [^{125}I]-labelled fibrinogen

All degradation reactions were conducted in duplicate. [^{125}I]-labelled fbg (1 or 2 mg, as indicated) was incubated (37°C) for various time points from 5 min to 20 hr with either conditioned medium (from 5x10^6 non-stimulated or PMA-stimulated neutrophils), neutrophil membranes (25 μg), neutrophil cytoskeleton (25 μg), lysosomal enzymes (from 5x10^6 neutrophils) or pure human neutrophil elastase (HNE) (2 μg) in a final volume of 1 ml HBSS, in the absence or presence of ethylene glycol tetra acetic acid (EGTA, 10 mM). [^{125}I]-labelled fbg (2 mg) was also incubated (37°C) for various time points from 2 min to 60 min with non-stimulated or PMA-stimulated neutrophils (5x10^6) in a final volume of 1 ml HBSS, in the presence or absence of EGTA (10 mM). When required, the concentration of the enzyme source was varied (see results). [^{125}I]-labelled fbg (1 mg) was degraded (37°C) with plasmin generated by the addition of porcine plasminogen (0.5 μg) and urokinase (4 iu, human kidney cells) in a final volume of 1 ml Tris (10

mM, pH 8.1) containing NaCl (140 mM) and either $CaCl_2$ (2 mM) or EGTA (10 mM). Fbg degradation products, generated by neutrophil conditioned medium were also degraded further with plasmin. The neutrophil conditioned medium from $5x10^6$ PMA-stimulated neutrophils was incubated (37°C, 18 hr) with [^{125}I]-labelled fbg (2 mg) whereafter porcine plasminogen (0.5 µg) and urokinase (4iu, human kidney cells) in a final volume of 1 ml Tris (10 mM, pH 8.1) containing $CaCl_2$, was added (37°C, 30 min). Degradation reactions containing the enzyme source were also preincubated for 15 min at 37°C with inhibitors prior to the addition of substrate (see results). At specific times (see results) 10% (w/v) TCA-soluble [^{125}I]-labelled fbg peptide formation was monitored (as for CRP degradation, see 3.4.2.). For visual analysis of [^{125}I]-labelled fbg degradation, aliquots of degradation reaction mixtures, at various time points, were mixed with an equal volume of SDS-sample buffer (0.012 M Tris [ph 6.8] with 4% [w/v] SDS and 24% [v/v] glycerol with or without 10% [v/v] 2-mercaptoethanol) and analysed by 5-20% SDS-PAGE (non-reduced) and 10% SDS-PAGE (reduced) (Laemmli, 1970). The gels were dried immediately and the products visualised by autoradiography. A kit containing pre-stained molecular weight markers of 14-200 kDa were used as calibration standards. When necessary the reduced 10% gels were stained for carbohydrate using a Periodic acid-Schiff base reagent (PAS) kit (Mantle and Allen, 1978) prior to autoradiography.

3.5.1.1. Two-dimensional iso-electric focusing of fibrinogen degradation products

[^{125}I]-labelled fbg degradation products generated by neutrophil conditioned medium at 20 hr as well as plasmin at 20 hr (as described above) were dialysed against water overnight (4°C), freeze dried and resuspended at 4 mg/ml in 20 mM Tris (pH 8.3), 1% (w/v) sodium decyl sulphate, and 7 M Urea (Shephard *et al.*, 1989). Aliquots of 80 µg of both the neutrophil conditioned medium and the plasmin generated fbg degradation products were loaded onto a prefocused Ampholine PAG plate (5% polyacrylamide with 3% crosslinkage and 2.2% [w/v] ampholine), (Pharmacia, Uppsala, Sweden) with a broad pH range between 3.5 and 9.5. An isoelectric calibration kit (Pharmacia, Uppsala, Sweden) containing standards with a pH range of 3.5-9.3 was included. This kit contains amyloglucosidase (pH 3.5), soybean trypsin inhibitor (pH 4.55), β-lactoglobin A (pH 5.2), bovine carbonic anhydrase B (pH 5.85), human carbonic anhydrase B (pH 6.55), horse myoglobin (pH 6.85), horse myoglobin (pH 7.35) lentil lectin (pH 8.15), lentil lectin (pH 8.45), lentil lectin (pH 8.65) and trypsinogen (pH 9.3). The proteins were focused on the PAG plate with a constant power of 15 watts, 1 500 volts and 25 milliAmps at 10°C. The iso-electro focused plates were either stained with Coomassie (0.12%), left to dry between two sheets of polyester film and

autoradiographed or they were subjected to second dimension SDS-PAGE (5-20% non-reduced) (Laemmli, 1970). In order to load the iso-electro focused protein onto the SDS-PAGE the PAG plate was inserted above the polyacrylamide gel and sealed with 1% low melting agarose in SDS-sample buffer (0.004 M Tris [pH 6.8] with 4% [w/v] SDS and 24% [v/v] glycerol). Fbg and fbg degradation product standards were loaded onto filter paper wicks which were similarly inserted above the polyacrylamide gel and sealed as described above. The gels were dried immediately and autoradiographed.

3.5.2. Determination of the molecular size of the neutrophil fibrinogen-degrading protease by SDS-PAGE analysis

Neutrophil membranes (200 μg), cytoskeleton (200 μg) and lyophilised conditioned medium from $50x10^6$ PMA-stimulated neutrophils were analysed separately by 3-13% SDS-PAGE (non-reduced) (Laemmli, 1970). The SDS was removed from the gel (as described above in section 3.4.4.1.) and enzyme activity was determined by incubating (37°C, 24 hr) 2 mm gel slices with [^{125}I]-labelled fbg (1 mg) in HBSS (1 ml) and measuring the generation of 10% (w/v) TCA-soluble [^{125}I]-labelled fbg peptides (as described in section 3.4.2. for CRP degradation).

3.5.3. Functional assessment of the fibrinogen degradation products

3.5.3.1. Thrombin-induced clotting time

Fbg degradation products were generated by incubating (37°C) [^{125}I]-labelled fbg (1 mg) with, either the conditioned medium from PMA-stimulated neutrophils ($5x10^6$), neutrophil cytoskeleton (25 μg), neutrophil membrane (25 μg) or plasmin, over a period of 5 min to 20 hr (see 3.5.1.). The thrombin induced clotting time of these [^{125}I]-labelled fbg degradation products was determined by the addition of 10 μl thrombin (bovine plasma, 2 iu/10 μl), to 100 μl aliquots of the fbg degradation products or intact [^{125}I]-labelled fbg to which 5 μg α_1AT and 100 iu/ml aprotinin had been added (5 min, 37°C) to stop degradation. From a dose response study using intact [^{125}I]-labelled fbg, a 50% reduction in functional [^{125}I]-labelled fbg extended the thrombin induced clotting time to 35 sec. The sample was considered uncoagulable if clotting had not occurred in 3 min.

3.5.3.2. Generation of anticoagulants

In order to determine anticoagulant activity of the fbg degradation products, 100 µl aliquots of the fbg digests, generated as described in 3.5.3.1. and to which 5 µg α_1AT and 100 iu/ml aprotinin had been added (5 min, 37°C) to stop degradation, were mixed with 100 µl intact fbg (3 mg/ml HBSS) and thrombin (1 iu/10 µl). The thrombin induced clotting time was measured, as described in 3.5.3.1., and anticoagulant activity was assessed by the prolongation of the clotting time.

3.5.3.3. Crosslinked fibrin formation by degraded fibrinogen

Intact [^{125}I]-labelled fbg and fbg degradation products, as generated in 3.5.3.1. from [^{125}I]-labelled fbg, were treated with 5 µg solid α_1AT followed by thrombin (1 iu/10 µl), incubated at 37°C for 3 min and then solubilised in an equal volume of 20 mM Tris (pH 8.3), 1% (w/v) sodium decyl sulphate, 7 M urea and 5% (v/v) 2-mercaptoethanol (Shephard *et al.*, 1989). The nature of the [^{125}I]-labelled fibrin and the formation of γ-γ dimers was then analysed on 10% SDS-PAGE (Laemmli, 1970) followed by autoradiography after the addition of an equal volume of SDS sample buffer (0.012 M Tris [pH 6.8] with 4% [w/v] SDS and 24% [v/v] glycerol with 5% (v/v) 2-mercaptoethanol.

3.5.4. Production of fibrinogen peptides and separation by h.p.l.c.

Unlabelled fbg (2 mg) was digested with neutrophil conditioned medium (from 5x10^6 PMA-stimulated neutrophils), neutrophil cytoskeleton (25 µg) and neutrophil membrane (25 µg) for various time periods (see above), in a final volume of 1 ml HBSS. When required the degradation was allowed to proceed to 20 hr and either stopped at this point or thrombin (2 iu/ml) was added to the mixture and the incubation was continued for a further 1 hr at 37°C. Unlabelled fbg (2 mg) was also digested with 2 µg pure HNE in a final volume of 1 ml HBSS. All reactions were stopped at the appropriate times by the addition of 50% (w/v) TCA to a final concentration of 10% (w/v) and the precipitated protein was removed by centrifugation (400 g for 10 min at 4°C). The TCA was extracted from the soluble peptide mix four times with four volumes of diethyl ether. The supernatant fraction was then freeze dried and resuspended in pyrogen free distilled H_20 (10% of the original volume). As required, aliquots of the peptide mix were adjusted to pH 7.4 with 0.1M NaOH, then incubated (37°C) with thrombin (2 iu/ml) or pure HNE (2 µg/ml) for 2 hr. Remaining high molecular mass protein was removed from this digest using a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) equilibrated with 0.1% trifluoroacetic acid. The adsorbed peptides were eluted with 75% acetonitrile in 0.1% trifluoroacetic acid (3 ml). After evaporation of the solvent, the peptides were redissolved in 0.1% trifluoroacetic acid and subjected to h.p.l.c. analysis.

The peptides were separated using a Merck Hibar Column (250x4 mm) prepacked with LiChrosorb RP-18 (5 μM, E. Merck, Darmstadt, Germany). H.p.l.c. was performed using a Waters Associates liquid chromatograph equipped with a model 600E controller, solvent delivery system (model 6000), automatic injector (model 710B), variable wave length absorbance detector (model 484 set at 220 nm), and a data module (model M730). The column was loaded with 100 μl peptide aliquots and eluted using a binary gradient formed from 0.1% trifluoroacteic acid (solution A) and 0.1% trifluoroacetic acid in acetonitrile/water (75:25, solution B). The initial condition of 95% solution A and 5% solution B run isocratically for 7 min was followed by a linear gradient for a further 53 min to 30% solution A and 70% solution B at a flow rate of 1 ml/min.

3.5.5. Amino acid analysis

Peptides for amino acid analysis were collected and further purified, then subjected to 6N HCL hydrolysis, in vacuo, for 24 hr. Amino acids were separated using the Waters h.p.l.c. amino acid analysis protocol employing a CXpak strong cation exchange column and detected by *o*-phthaldialdehyde (OPA) derivatisation. When proline was determined, the OPA-hypochlorite detection system was used.

3.5.6. Association of [^{125}I]-labelled fibrinogen with neutrophils and concomitant degradation

[^{125}]I-labelled fbg (1mg) was incubated (37^{o}C) for various time points from 2 to 60 min with 5x10^{6} non-stimulated and PMA (5 ng/ml)-stimulated neutrophils in a final volume of 1 ml HBSS. In order to assess the effect of Ca^{2+} on the association of fbg with PMA-stimulated neutrophils, PBS was used as a Ca^{2+} depleted incubation buffer. A concentration curve of [^{125}I]-labelled fbg (from 0-1mg) with non-stimulated and PMA (5 ng/ml)-stimulated neutrophils (5x10^{6}) in the absence and presence of AEBSF (1mM) was also set up. Prior to the addition of [^{125}I]-labelled fbg (1mg), PMA stimulated neutrophils (5x10^{6}) were also preincubated (37°C) for 15 min with saturating concentrations (as determined by flow cytometry) of monoclonal antibodies to CD16 (Sanbio 1041C), CD11a (Sanbio 1020, Serotec), CD11b (Sanbio 1019, OKM1 and OKM10), CD11c (Sanbio 1011, Serotec), CD18 (Sanbio 1110, Serotec), and CD41a (Sanbio 1144 and Sanbio 1145 directed against Fc dependent reactions and fbg binding sites on platelets respectively). When the effect of monoclonal antibody and AEBSF was investigated, AEBSF (1 mM) was preincubated (15 min at 37^{o}C) with 5x10^{6} neutrophils prior to the addition of the

antibody, whereafter a further incubation period (15 min at 37°C) was allowed prior to the addition of [^{125}I]-labelled fbg (1mg). In order to assess the effect of fbg peptides on neutrophil association of fbg, the peptides were prepared, as described in 3.5.4., by the action of neutrophil conditioned medium and adjusted to pH 7.4. These neutrophil-derived fbg peptides as well as the synthetic fbg peptides RGDS (50 µg), H10 (50 µg), H12 (50 µg) and H15 (50 µg), were preincubated (37°C for 20 min) with 5x10^6 PMA stimulated neutrophils, prior to the addition of [^{125}I]-labelled fbg (1mg). Degradation reactions containing non-stimulated or PMA-stimulated neutrophils were also preincubated (37°C, 15 min) with inhibitors, prior to the addition of substrate (see results). All reactions were carried out in duplicate. At specific time points (see results) the association of [^{125}I]-labelled fbg with the cells was assessed by washing the neutrophils three times with incubation buffer (HBSS) at 4°C, then transferring the cells to clean tubes and monitoring the [^{125}I]-labelled fbg bound to the neutrophils in a gamma counter. Non-specific binding was determined in the presence of a 100 fold molar excess of unlabelled fbg. Specific binding was calculated by subtracting the non-specific binding. The nature of neutrophil associated [^{125}I]-labelled fbg, in the absence of AEBSF, was investigated by mixing aliquots of the washed neutrophil suspension with an equal volume of SDS-sample buffer (0.012 M Tris [pH 6.8], 4% (w/v) SDS and 24% (v/v) glycerol) and analysing by 5-20% SDS-PAGE (Laemmli, 1970). Gels were dried immediately and subjected to autoradiography. Degradation of the [^{125}I]-labelled fbg, during the course of these binding studies, was monitored by TCA (10%, w/v)-soluble peptide formation (see 3.5.1). The duplicates did not differ by more than 10% from each other and the mean of the duplicates was calculated.

3.5.7. Influence of fibrinogen on the release of fibrinogen degrading activity from the neutrophil

[^{125}I]-labelled fbg (1mg) was incubated (37°C) with PMA (5 ng/ml)-stimulated neutrophils (5x10^6). At specific time points (see results) the cells were removed by centrifugation (400 g for 3 min) and the extracellular medium returned to 37°C. Degradation of [^{125}I]-labelled fbg in the extracellular medium was assessed and compared, at specific matching time points, with that of degradation of [^{125}I]-labelled fbg (1mg) by PMA stimulated neutrophils (5x10^6) or by neutrophil conditioned medium, prepared in the absence of fibrinogen, from 5x10^6 cells. Degradation was monitored by 10% (w/v) TCA-soluble peptide formation (see 3.5.1.).

3.5.8. The influence of low molecular weight fibrinogen peptides on the association of [^{51}Cr]-labelled neutrophils to solid phase fibrinogen

Falcon 3911 Microtest III (Falcon Labware, Becton-Dickinson, Oxnard, CA) 96-well plates were coated with 250 µl aliquots of fbg (1 mg/ml PBS) at 4°C overnight. The wells were washed three times with PBS, blocked with PBS containing 1% BSA for 2 hr at room temperature and finally washed three times with HBSS and used immediately. Neutrophils ($50x10^6$) were incubated (37°C) with $Na_2[^{51}Cr]O_3$ (100 µCi) for 1 hr and subsequently washed three times with HBSS to remove unincorporated [^{51}Cr]. Prior to the addition of PMA (5 ng/ml) TCA (10%, w/v)-soluble fbg peptides (prepared by degrading fbg with neutrophil conditioned medium for 9 hr, as described above in 3.5.4.), at a concentration range of 0-100 µg, were preincubated (30 min at 37°C) with $2x10^6$ [^{51}Cr]-labelled neutrophils in a final volume of 1 ml HBSS. Immediately following stimulation with PMA, quadruplicate 100 µl aliquots were transferred to the fbg coated wells and incubated (37°C) with the solid phase fbg for 30 min. The non-adherent cells were removed by washing the wells three times with HBSS. Individual wells were cut from the plate and counted in a gamma counter in order to quantify the adherent cells. Quadruplicates did not differ by more than 10% and the mean of the quadruplicates was calculated. The number of adherent cells in the presence of the fbg degradation products was expressed as a percentage of the total number of adherent cells in the absence of these products.

CHAPTER 4

INTERACTION OF CRP WITH NEUTROPHILS

RESULTS AND DISCUSSION

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4.A. C-REACTIVE PROTEIN ASSOCIATION WITH NEUTROPHILS AND CONCOMITANT DEGRADATION

4.A.1. INTRODUCTION

There are numerous reports indicating the interaction of [^{125}I]-labelled CRP with neutrophils via a receptor-mediated process (Muller and Fehr, 1986; Buchta *et al.*, 1987a; Shephard *et al.*, 1986). However, a study investigating the integrity of CRP following association with neutrophils revealed considerable neutrophil-mediated degradation of CRP which generated both high molecular weight CRP intermediates and low molecular weight TCA-soluble CRP peptides. The study also showed, with the use of SDS-PAGE, that the neutrophil-associated CRP was comprised of both intact CRP and the CRP degradation products (Shephard *et al.*, 1989). There was a positive correlation between the extent of degradation and the association of CRP and CRP intermediates with the neutrophil. This association of CRP degradation products with the cell was suggested by Shephard *et al.* (1989) to account for the high, non-specific binding of CRP to neutrophils, observed in previous studies (Muller and Fehr, 1986; Buchta *et al.*, 1987a). Neutrophil association of both CRP and CRP degradation products was enhanced two fold when the cells were stimulated with PMA at 10 ng/ml., a concentration of PMA that did not cause significant release from the azurophil granules (Shephard *et al.*, 1989; Pontremoli *et al.*, 1986). Inclusion of calcium in the incubation of CRP with PMA-stimulated neutrophils caused a 25% to 40% decrease in the total neutrophil associated CRP, although the ratio of associated CRP to CRP degradation products remained the same as that in the absence of calcium. This modulation of CRP association with neutrophils by calcium was suggested to be due to a calcium dependent conformational change in the CRP molecule (Shephard *et al.*, 1989).

The generation of CRP degradation products was concluded to occur as a result of the action of a PMA-upregulatable neutrophil membrane-associated neutral protease during the association of CRP with the cells (Shephard *et al.*, 1989).

The aim of this study was to investigate the interaction of CRP with neutrophils in the presence of inhibitors of neutrophil-mediated degradation of CRP in the hope of identifying the process of CRP association with the neutrophil and the existence of a possible CRP receptor on the neutrophil membrane.

4.A.2. RESULTS

4.A.2.1. Influence of AEBSF, in the presence and absence of Ca^{2+}, on neutrophil association and degradation of CRP

PMA-stimulated neutrophil association and degradation of [^{125}I]-labelled CRP was investigated in the presence and absence of the serine protease inhibitor AEBSF. AEBSF (1 mM) was shown to inhibit the degradation of 20 μg [^{125}I]-labelled CRP by PMA-stimulated neutrophils ($10x10^6$), by 98% at 30 min. In the absence of AEBSF 706 ng [^{125}I]-labelled TCA-soluble CRP peptides were generated and in the presence of AEBSF 14 ng [^{125}I]-labelled TCA soluble peptides were formed.

In the absence of AEBSF the association of CRP with PMA stimulated neutrophils was shown to occur as early as 2 min and reached a steady state between 30 and 40 min (not shown). This confirmed previous results of Shephard *et al.* (1989). Concentration dependence curves, in the absence of AEBSF (Fig 5) also confirmed previous results that saturation could not be reached. With 100 μg [^{125}I]-labelled CRP offered and in the absence of Ca^{2+}, 860 ng [^{125}I]-labelled CRP associated with PMA-stimulated neutrophils ($10x10^6$) in a final volume of 1 ml at 30 min (Fig 5). With the addition of Ca^{2+} to this reaction a decrease in the total neutrophil associated [^{125}I]-labelled CRP by approximately 50%, was observed (Fig 5).

In the presence of AEBSF (1 mM) the association of [^{125}I]-labelled CRP with PMA-stimulated neutrophils was inhibited by 55-58% when compared with that in the absence of AEBSF (Fig 5), but the association appeared to be saturable (Fig 5). With 100 μg [^{125}I]-labelled CRP offered to $10x10^6$ neutrophils in the absence of Ca^{2+}, 360 ng [^{125}I]-labelled CRP was associated with the cells after 30 min (Fig 5). Half maximal saturation occurred at 20 μg [^{125}I]-labelled CRP/ml (Fig 5). In the presence of Ca^{2+}, 208 ng [^{125}I]-labelled CRP (100 μg offered) was found to associate with $10x10^6$ PMA-stimulated neutrophils within 30 min. Half maximal saturation was found to occur at 40 μg [^{125}I]-labelled CRP/ml (Fig 5). Ten different experiments gave results within 10% of these.

The association of [^{125}I]-labelled CRP (20 μg offered) with 10x10^6 PMA-stimulated neutrophils at 30 min, in the presence of AEBSF, (1 mM) (Fig 5) could not be inhibited by an excess of unlabelled CRP, either in the absence or presence of Ca^{2+} (Table 2). The association of [^{125}I]-labelled CRP (20 μg offered) with 10x10^6 neutrophils at 30 min, in the absence of Ca^{2+} (140 ng/10x10^6 cells, Fig 5) was increased to 220 ng/10x10^6 cells/ 30 min (in the presence of 5 mg unlabelled CRP), while that in the presence of Ca^{2+} (60 ng/10x10^6 cells, Fig 5), was increased to 90 ng/10x10^6 cells/ 30 min (Table 2). Neutrophil associated [^{125}I]-labelled CRP (20 μg offered) in the absence of Ca^{2+} (140 ng/10x10^6 cells/30 min, Fig 5) was increased to 164 ng in the presence of 1 mg of unlabelled CRP and to 75 ng from 60 ng/10x10^6 cells/30 min (Fig 5), in the presence of Ca^{2+} (Table 2).

Table 2. Infuence of unlabelled CRP on the association of [^{125}I]-labelled CRP with neutrophils, in the presence of AEBSF with or without Ca^{2+}. PMA-stimulated neutrophils (10x10^6) were incubated (37°C, 30 min) with [^{125}I]-labelled CRP (20 μg) in the presence of AEBSF (1mM), with or without Ca^{2+}. Unlabelled CRP of either 5 mg or 1 mg was included in the incubation. Association of [^{125}I]-labelled CRP is expressed as ng/10x10^6 neutrophils at 30 min.

	ng [^{125}I]-labelled bound CRP/10x10^6 cells	
unlabelled CRP	+Ca^{2+}	–Ca^{2+}
none	60	140
5 mg	90	220
1 mg	75	164

SDS-PAGE (5-20% non-reduced) (Fig 6) of the cell-associated [^{125}I]-labelled CRP in the presence of AEBSF and in both the absence and presence of Ca^{2+} at 30 min, clearly showed there to be more [^{125}I]-labelled CRP associated with the cell in the presence of the unlabelled CRP than in its absence. The extent of [^{125}I]-labelled CRP binding to neutrophils in the presence of unlabelled CRP appeared to be related to the concentration of unlabelled CRP in the incubation (Table 2, Fig 6). A 21 kDa degradation product, in addition to the CRP subunit (24 kDa) could be detected in association with the cells (Fig 6).

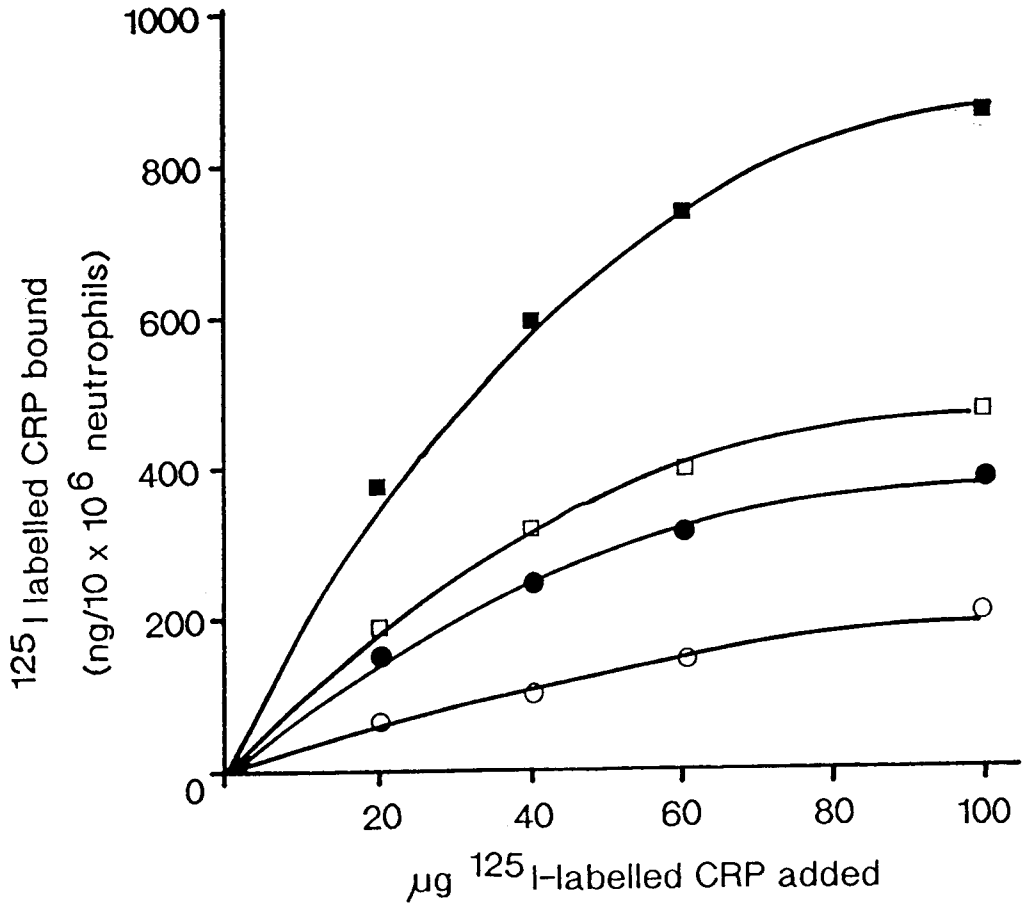

Figure 5. Association of [^{125}I]-labelled CRP with PMA stimulated neutrophils as a function of concentration. [^{125}I]-labelled CRP (0-100 µg) was incubated (37°C, 30 min) with PMA-stimulated neutrophils ($10x10^6$) in the presence of the inhibitor AEBSF (1 mM), with (○) or without calcium (●), and in the absence of AEBSF, with (□) or without calcium (■). Association of [^{125}I]-labelled CRP was measured and expressed as ng [^{125}I]-labelled CRP/$10x10^6$ cells. Each value is a mean of duplicates which did not differ by more than 10%. Ten experiments gave results within 10% of these.

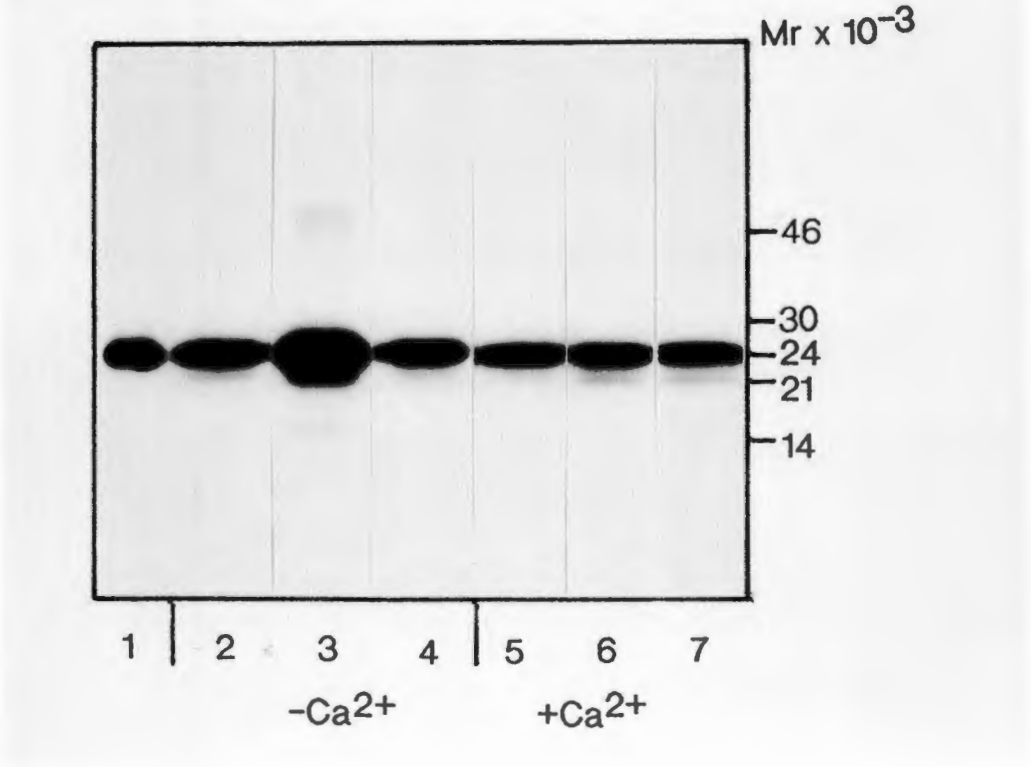

Figure 6. SDS-PAGE of neutrophil associated [^{125}I]-labelled CRP. [^{125}I]-labelled CRP (20 μg) was incubated (37°C, 30 min) with 10x10^6 PMA-stimulated neutrophils in the absence and presence of Ca^{2+} and in the presence of a 250 fold or 50 fold excess of unlabelled CRP. The neutrophils were washed at the end of the incubation period and analysed on 5-20% SDS PAGE (non-reduced) as described in the Materials and Methods. Track 1 represents an undigested CRP standard. Neutrophil associated [^{125}I]-labelled CRP in the absence of unlabelled CRP is indicated in tracks 2 and 5 (without and with Ca^{2+}, respectively), while tracks 3 and 6 represent neutrophil associated [^{125}I]-labelled CRP in the presence of a 250 fold excess of unlabelled CRP and in the absence and presence of Ca^{2+}, respectively. Tracks 4 and 7 show the association of [^{125}I]-labelled CRP to PMA-stimulated neutrophils in the presence of a 50 fold excess of unlabelled CRP and in the absence and presence of Ca^{2+}, respectively.

4.A.1.2.4. Influence of monoclonal antibodies to neutrophil integrins on neutrophil association and degradation of [^{125}I]-labelled CRP

The influence of monoclonal antibodies to neutrophil integrin receptors, on the neutrophil association and degradation of [^{125}I]-labelled CRP was pursued. Saturating concentrations of the monoclonal antibodies directed against CD11a, CD11b, CD11c, CD18, CD16 and the gpIIb/IIIa complex (CD41a) were determined by flow cytometry. These studies were conducted in the presence and absence of the inhibitor AEBSF, which, at a concentration of 1 mM was shown to inhibit [^{125}I]-labelled CRP degradation by 98%.

None of the monoclonal antibodies preincubated with PMA-stimulated neutrophils, in the absence or presence of AEBSF and the absence of Ca^{2+}, modulated the association of 360 ng [^{125}I]-labelled CRP with $10x10^6$ PMA-stimulated neutrophils at 30 min (20 μg [^{125}I]-labelled CRP offered) (data not shown).

A 90% inhibition of degradation of 20 μg [^{125}I]-labelled CRP by $10x10^6$ PMA-stimulated neutrophils at 30 min was observed in the presence of anti-CD11c (Serotec). The generation of 706 ng [^{125}I]-labelled TCA-soluble CRP peptides by $10x10^6$ cells at 30 min was thus reduced by 635 ng [^{125}I]-labelled TCA-soluble peptides when anti-CD11c was included in the incubation, in the absence of AEBSF. The inclusion of AEBSF (1 mM) in the reaction increased the inhibition of degradation to 100%. In addition, saturating concentrations of the monoclonal antibodies directed against CD11b (Sanbio), CD11a (Serotec) and CD18 (Sanbio) reduced the neutrophil-mediated degradation of [^{125}I]-labelled CRP (20 μg offered) at 30 min, in the absence of AEBSF, by 45%, 75% and 65%, respectively. However, in the presence of AEBSF, no further inhibition of [^{125}I]-labelled CRP degradation was observed with any of these monoclonal antibodies. No inhibition of [^{125}I]-labelled CRP degradation was observed with the monoclonal antibodies directed against CD16 (Sanbio) and CD41a (Sanbio), in the absence or presence of AEBSF. Two different monoclonal antibodies directed against two different epitopes of CD41a gave the same result. Due to the complete absence of inhibition of [^{125}I]-labelled CRP degradation by the antibodies directed against CD16 and CD41a, these antibodies were treated as negative controls.

Due to the expense of monoclonal antibodies, experiments showing saturation curves of neutrophil association of [^{125}I]-labelled CRP, in the presence of both AEBSF and anti-CD11c where 100% inhibition of degradation was shown, were not possible. However, SDS-PAGE analysis of the neutrophil associated [^{125}I]-labelled CRP at 30 min, in the presence of AEBSF and anti-CD11c, showed it to be of subunit size (24 kDa) (not shown). This binding was not inhibitable in the presence of unlabelled CRP, but rather facilitation of [^{125}I]-labelled CRP binding to neutrophils, as demonstrated in Table 2, was observed. Association of the 21 kDa degradation product was not detectable.

4.A.3. DISCUSSION

Previous studies, which have attempted to identify the putative CRP receptor on the neutrophil, have reported high (50%) non-specific association of CRP with the neutrophil (Muller and Fehr, 1986; Buchta *et al.*, 1987a; Shephard *et al.*, 1989). In addition, degradation of CRP, during its association with neutrophils, has been shown to occur by a PMA-activatable membrane-associated protease and the CRP degradation products have been demonstrated to associate with the neutrophil surface (Shephard *et al.*, 1989). The demonstration of concomitant degradation of CRP during association with PMA-stimulated neutrophils thus precludes identification of a CRP receptor on the neutrophil membrane by Scatchard analysis of binding data.

This study re-examines the interaction of CRP with PMA-stimulated neutrophils in the presence of the inhibitor AEBSF, which inhibits neutrophil-mediated degradation of CRP by 98%. In accordance with previous results (Shephard *et al.*, 1989), saturation of CRP binding to neutrophils could not be reached in the absence of AEBSF. When neutrophil-mediated degradation of CRP was inhibited by AEBSF, the total association of CRP with the cell was saturable, but the total amount of CRP associated with the cell was less than that in the absence of this inhibitor. The presence of Ca^{2+} in the incubation medium, in both the absence and the presence of AEBSF, also influenced the total amount of CRP associated with the neutrophil. Thus, the conformation of CRP, which is calcium dependent Swanson *et al.*, 1991; Mullenix and Mortensen, 1994), governs the extent of interaction of CRP with neutrophils, as previously described (Buchta *et al.*, 1987a; Shephard *et al.*, 1989). SDS-PAGE analysis of the neutrophil associated material, in the presence of AEBSF with or without Ca^{2+}, revealed the association of CRP corresponding to the molecular mass of the CRP subunit (24 kDa) in addition to a CRP degradation product of 21 kDa. The generation of the 21 kDa product could reflect the inability of AEBSF to completely inhibit CRP degradation. From the autoradiographic analysis it appears that this 21 kDa product was less than 10% of the total CRP associated with the cell. The specificity of the interaction of CRP with the neutrophil, in the presence of AEBSF, could not, however, be deduced as no inhibition of the interaction of radiolabelled CRP with neutrophils could be achieved with unlabelled CRP, but rather facilitative binding was observed. The total association of [^{125}I]-labelled CRP with neutrophils appeared to be positively related to the concentration of unlabelled CRP in the incubation reaction.

The facilitation of radiolabelled CRP binding to neutrophils, in the presence of unlabelled CRP, could be due to CRP subunit binding to neutrophils rather than intact pentameric CRP binding to the neutrophil as SDS-PAGE analysis does not distinguish between pentameric and subunit CRP. The CRP subunit has, however, been found to be very hydrophobic and consequently if it is associated with the neutrophil membrane could facilitate further CRP binding. In addition, the 21 kDa degradation product could also facilitate CRP binding. A decrease in solubility of CRP has been noted upon its degradation or reduction into subunits with a resultant propensity for the products to aggregate (Bray *et al.*, 1987; Potempa *et al.*, 1987).

Thus, even though neutrophil-mediated degradation of CRP is inhibited by 98% by AEBSF and the association of CRP with neutrophils is saturable in the presence of this inhibitor, the inability of the association of radiolabelled CRP to be inhibited by unlabelled CRP, precludes Scatchard analysis of the binding data. However, the ability of the antibodies to the CD11c, CD11a, CD11b and CD18 neutrophil integrin receptors to inhibit neutrophil-mediated degradation of CRP suggests that the putative CRP receptor may be in close proximity to these receptors. Since this inhibition was greatest (90%) in the presence of anti-CD11c, the CRP receptor may be postulated to be closest to CD11c/CD18 as opposed to CD11b/CD18 or CD11a/CD18. The observed inhibition of CRP degradation by anti-CD11a, anti-CD11b and anti-CD18 may be due to obstruction of ligand interaction with the putative CRP receptor in the vicinity of CD11c, by these antibodies. We have shown that during the incubation of fibrinogen with neutrophils there is degradation of this ligand by the membrane-associated protease described here (see Chapter 5). We also showed that neutrophil-mediated degradation of fibrinogen could be inhibited with the antibody directed against CD11c. This supports the hypothesis that the interaction of CRP with the neutrophil membrane, which is a necessary requirement for neutrophil-mediated CRP degradation, occurs at a position in close proximity to the CD11c integrin receptor. Of significance, was the inability of the antibody directed against the IgG Fc receptor (CD16) to modulate neutrophil degradation or association of CRP, in the presence and absence of AEBSF. Previous reports have suggested the CRP receptor on the neutrophil to either be, or be in close proximity to, the IgG receptor (Buchta *et al.*, 1987a; Zeller and Sullivan, 1992; Zeller and Sullivan, 1993).

4.B. IDENTIFICATION OF THE CRP-DEGRADING PROTEASE AND THE MECHANISM OF CRP PROTEOLYSIS

4.B.1. INTRODUCTION

Previous studies looking at the interaction of CRP with neutrophils have established concomitant degradation of the ligand giving rise to large molecular weight products which become cell associated and small peptides which remain in the extracellular medium (Shephard *et al.*, 1989). An important consequence of this neutrophil-mediated degradation of CRP is the observation that the low molecular weight peptides are potent non-specific inhibitors of neutrophil migration and oxidative metabolism (Shephard *et al.*, 1989; Shephard *et al.*, 1990; Shephard *et al.*, 1992). The inhibitory activity of these peptides was shown to be related to the inhibition of the glycolytic enzyme, enolase, with subsequent inhibition of glycolysis followed by depletion in intracellular ATP levels (Shephard *et al.*, 1992).

Although non-stimulated neutrophils were able to degrade CRP, the rate of degradation was only minimally greater than that of neutrophil conditioned medium. When neutrophils were stimulated with PMA, a neutrophil-associated enzyme considerably enhanced the rate of CRP-degradation when compared with CRP degradation by PMA-stimulated neutrophil conditioned medium. Detection of CRP degradation products within 2 min of incubating CRP with PMA-stimulated neutrophils, led to the suggestion that neutrophil-mediated degradation of CRP is due to a neutrophil membrane-associated protease which is maximally activated by PMA. Further confirmation for this was obtained when it was shown that isolated neutrophil membranes produced CRP peptides with identical amino acid sequences as those generated by intact neutrophils (Shephard *et al.*, 1991). In addition, the inhibitor profile and nature of CRP products generated by isolated neutrophil membranes was the same as that of PMA-stimulated neutrophils (Shephard *et al.*, 1989).

This study attempts to further identify the neutrophil membrane-associated protease with respect to cellular distribution and molecular size. The mechanism of CRP degradation, with particular reference to the interaction of CRP with the neutrophil surface resulting in degradation, will be examined.

4.B.2. RESULTS

4.B.2.1. Identification of the CRP-degrading protease

4.B.2.1.1. Association of the CRP degrading enzyme with the neutrophil cytoskeleton

Proteins involved in the attachment of the cytoskeleton with the plasma membrane are found to co-isolate in both membrane and cytoskeleton fractions (Luna and Hitt, 1992). When a neutrophil Triton X-100 insoluble fraction, containing cytoskeleton proteins, was incubated with [^{125}I]-labelled CRP (100 μg), TCA-soluble [^{125}I]-labelled CRP peptides were produced in both a time and enzyme concentration dependent manner (Table 3). [^{125}I]-labelled CRP degradation products were observed on 12% SDS-PAGE (reduced) at apparent molecular mass values of 21 kDa and 18 kDa (Fig 7). These CRP degradation products, formed through the action of the neutrophil cytoskeleton fraction, were found to be identical in molecular mass to those generated by the action of the neutrophil membrane and the conditioned medium from PMA-stimulated neutrophils but different from those generated by the action of neutrophil lysosomal enzymes (Fig 7). Approximately 98% of the total CRP-degrading enzyme associated with the insoluble cytoskeleton was solubilised with 1M NaCl and the proteolytic activity remained in solution following removal of the 1 M NaCl by dialysis against PBS. Similar solubility of the membrane associated protease has been described by Shephard *et al.*(1989).

Table 3. Generation of [^{125}I]-labelled TCA-soluble CRP peptides by neutrophil cytoskeleton. [^{125}I]-labelled CRP (100 μg) was incubated with varying concentrations of neutrophil cytoskeleton and at various time points (2 hr-6 hr) the generation of 10% (w/v) TCA-soluble [^{125}I]-labelled CRP petides was measured and expressed in μg/ml.

	[^{125}I]-labelled TCA-soluble CRP peptides (μg/ml)		
	Time (hr)		
Cytoskeleton (μg)	2	4	6
10	10	18	20
50	14	25	35
100	22	40	55

The ability of the neutrophil cytoskeleton fraction to degrade CRP was inhibited by the serine protease inhibitor PMSF (1mM), the elastase inhibitor α_1AT (1.5 mg/ml) (Fig 7), the chymotrypsin inhibitor L-1-tosylamido-2-phenyl-ethyl-chloromethyl-ketone (TPCK, 5μM) and the trypsin inhibitor α_1-p-tosyl-L-lysine-chloromethyl-ketone (TLCK, 10 μM). These inhibitors were shown previously to be equally capable of inhibiting the CRP-degrading activity associated with the neutrophil membrane (Shephard *et al.*, 1989). Inhibition of the cytoskeleton associated enzyme by these inhibitors was such that less than 3 μg of TCA-soluble CRP peptides were formed when they were present during the incubation of CRP with neutrophils.

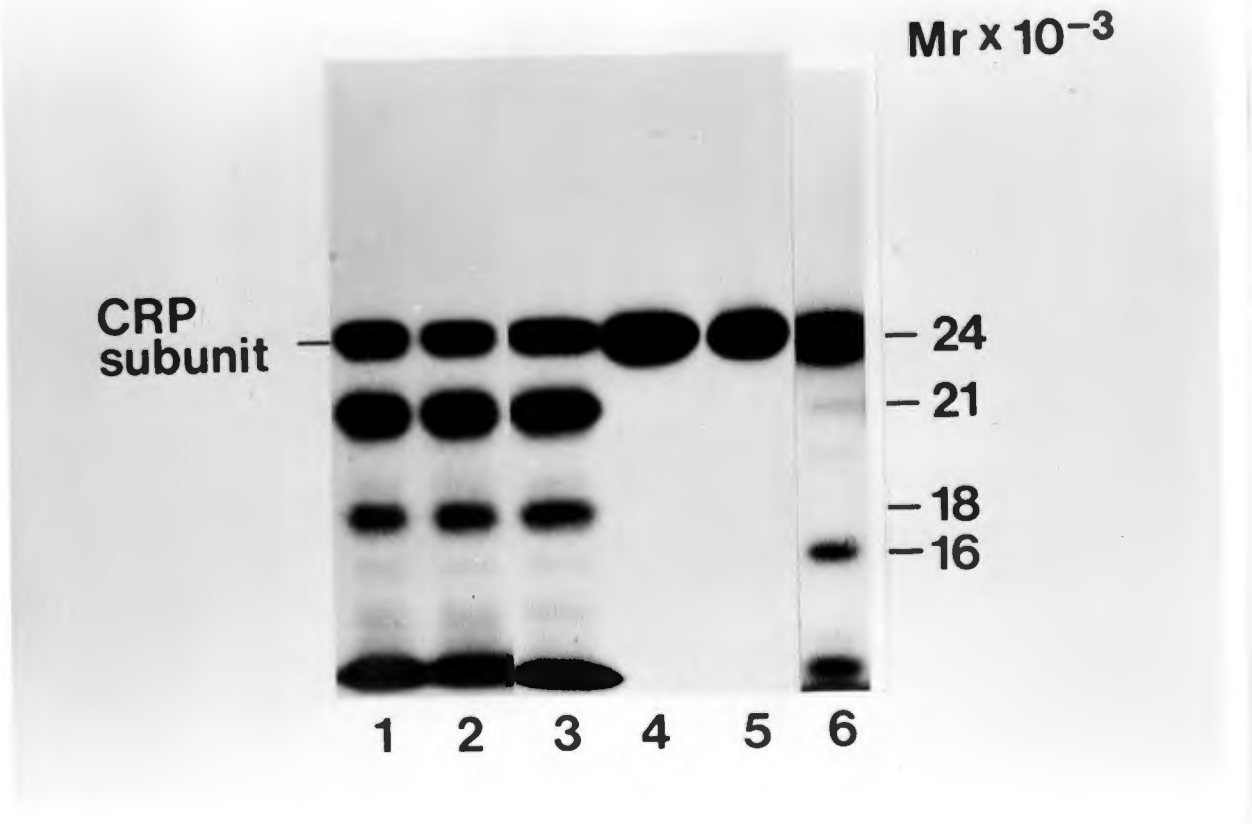

Figure 7. SDS-PAGE (12% reduced) of [^{125}I]-labelled CRP degradation products. [^{125}I]-labelled CRP (100 μg) was degraded (37°C, 1 hr) by either, 100 μg neutrophil cytoskeleton (Track 1), 100 μg neutrophil membranes (Track 2), conditioned medium from 10x10^6 PMA-stimulated neutrophils (Track 3) or the lysosomal enzymes from 5x10^6 neutrophils (Track 6). Preincubation (37°C, 15 min) of the inhibitors PMSF and α_1AT with the neutrophil cytoskeleton, resulted in inhibition of [^{125}I]-labelled CRP degradation (Tracks 4 and 5, respectively).

4.B.2.1.2. Molecular size of the CRP-degrading protease

This was ascertained by SDS-PAGE using non-reducing conditions and by gel filtration.

SDS-PAGE

The proteins in the neutrophil cytoskeleton fraction (200 μg), membrane fraction (200 μg) and PMA-stimulated neutrophil conditioned medium (from 100x10^6 neutrophils) were separated by 3-13% SDS-PAGE (non-reduced). To determine which proteins possessed proteolytic activity the gel was cut into 2 mm slices which were then incubated with [^{125}I]-labelled CRP and assayed for CRP-degrading activity. Each enzyme preparation was identified to contain four distinct protein bands which were capable of degrading [^{125}I]-labelled CRP. For all three neutrophil fractions

about 1%, 5%, 70% and 20% of the total loaded CRP-degrading activity was detected in the gel slices containing molecules migrating to apparent molecular mass values of 501 kDa, 398 kDa, 316 kDa and 209 kDa respectively. These four proteolytic components of the neutrophil cytoskeleton, membrane and conditioned medium could not, however, be visualised on 3-13% SDS-PAGE (reduced and non-reduced) with coomassie staining (not shown). The three neutrophil fractions (150 μg) were subsequently labelled with [^{125}I]-Na (150 μCi) and the proteins separated by 3-13% SDS-PAGE followed by autoradiographic analysis whereupon the proteolytically active bands could be visualised at the corresponding molecular mass values of 501 kDa, 398 kDa, 316 kDa and 209 kDa (Fig 8). None of the other bands visualised with autoradiographic analysis were shown to possess proteolytic activity (Fig 8).

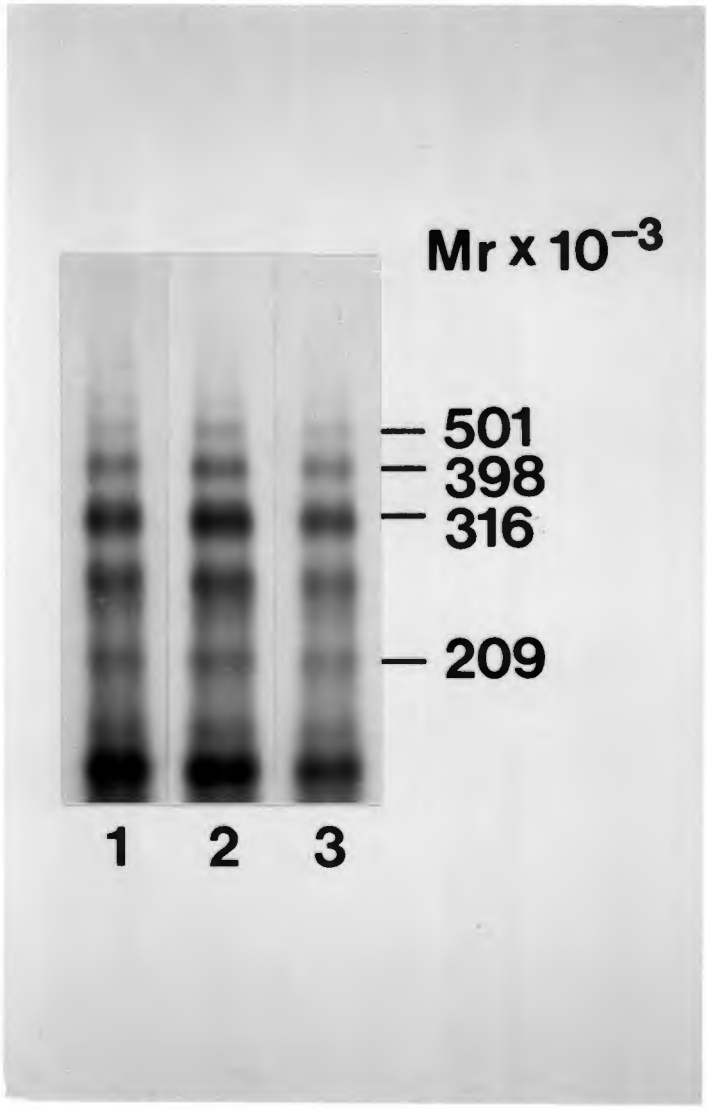

Figure 8. Molecular size of the CRP-degrading protease. SDS-PAGE (3-13% non-reduced) followed by autoradiography of [^{125}I]-labelled neutrophil membranes (200 μg) (Track 1), [^{125}I]-labelled neutrophil cytoskeleton (200 μg) (Track 2) and [^{125}I]-labelled conditioned medium from PMA-stimulated neutrophils ($100x10^6$) (Track 3) revealed four bands possessing CRP-degrading activity (Materials and methods). These bands migrated to the apparent molecular mass values of 501 kDa, 398 kDa, 316 kDa and 209 kDa, as indicated.

SIZE-EXCLUSION CHROMATOGRAPHY

The neutrophil cytoskeleton (500 μg) and membrane (500 μg) proteins and the conditioned medium from $100x10^6$ PMA-stimulated neutrophils were individually subjected to gel filtration using Sephacryl S-300 equilibrated in PBS. Greater than 95% of the proteolytic activity of each of these three fractions eluted in a single peak with an apparent molecular mass of 600 kDa (Table 4).

Table 4. Determination of the molecular mass of the CRP-degrading protease in isolated neutrophil membrane and cytoskeleton fractions and the conditioned medium from PMA-stimulated neutrophils by Sephacryl S-300 chromatography. The neutrophil membrane and cytoskeleton fractions (from $100x10^6$ cells) and the PMA-stimulated neutrophil conditioned medium (from $100x10^6$ cells) were loaded onto the Sephacryl S-300 column (1x45 cm) in PBS and eluted at 3ml/hr in 0.6 ml fractions. CRP-degrading protease activity was ascertained by incubating (37°C, 18 hr) 0.5 ml aliquots of each fraction with 100 μg [^{125}I]-labelled CRP. Degraded CRP is measured as the generation of 10% (w/v) TCA-soluble peptides and is expressed as μg [^{125}I]-labelled CRP/0.5 ml. The molecular mass of the protease in the peak fraction was calculated from the standards (used to calibrate the column) as indicated. The y-intercept was calculated as 1.874, the slope as –0.3122 and r as –0.978. An average of the molecular mass of the protease within the three eluted peak fractions was calculated.

	Degraded CRP (μg/0.5 ml)				
Column fraction	Membrane	Cytoskeleton	Conditioned Medium	K_{av}	kDa
1-28	0	0	0		
29	2	1	1	0.053	691
30	16	25	22	0.080	588 593
31	24	30	25	0.107	501
32	2	1	1		
33	0	0	0		
34	0	0	0		
35	0	0	0		
36	0	1	0		
37-60	0	0	0		
Protein Standards	Thyroglobulin			0.052	669
	Catalase			0.190	210
	Aldolase			0.300	158
	Bovine serum albumin			0.342	67
	Ovalbumin			0.434	43

When this eluted peak, from all three fractions, was labelled with [^{125}I]-Na, subjected to analysis by 3-13% SDS-PAGE (non-reduced) followed by autoradiography, four bands could be seen at the apparent molecular masses 501 kDa, 398 kDa, 316 kDa and 209 kDa (as in Fig 8). When the technique described above to recover proteolytic activity after SDS-PAGE was used, approximately 7%, 15%, 60% and 18% of the total loaded CRP-degrading activity could be recovered from the gel slices containing proteins which migrate to apparent molecular mass values 501 kDa, 398 kDa, 316 kDa and 209 kDa respectively.

4.B.2.2. Mechanism of CRP Proteolysis

4.B.2.2.1. Influence of [^{125}I]-labelled CRP on the proteolytic activity of the extracellular medium during cellular degradation

Degradation of [^{125}I]-labelled CRP by PMA-stimulated neutrophils was markedly faster than that by PMA-stimulated neutrophil-conditioned medium (Table 5). Experiments were conducted to ascertain whether the presence of CRP itself may effect the release of proteolytic activity from the neutrophil and subsequently its own degradation in the extracellular medium. To accomplish this, [^{125}I]-labelled CRP was incubated with PMA-stimulated neutrophils for various time periods whereupon the cells were removed and the cell-free medium restored to 37^{o}C to allow the degradation of extracellular CRP to continue. Upon removal of the cells from the reaction the [^{125}I]-labelled CRP in the cell-free medium continued to degrade. When the incubation period of [^{125}I]-labelled CRP with the cells was less than 40 min the rate of [^{125}I]-labelled CRP degradation in the extracellular medium was slower than the rate of [^{125}I]-labelled CRP degradation in the presence of PMA-stimulated cells but faster than that by PMA-stimulated neutrophil-conditioned medium (Table 5). When the incubation period of [^{125}I]-labelled CRP with the PMA-stimulated cells was greater than 40 min, the rate of [^{125}I]-labelled CRP degradation in the extracellular medium was similar to that in the presence of PMA-stimulated cells (Table 5). This CRP mediated release of proteolytic activity from the neutrophil was found to be concentration dependent and optimal at a concentration of 500 μg CRP/10x10^{6} cells.

CRP was also shown to mediate the release of CRP-degrading activity from non-stimulated neutrophils. As for stimulated cells, a 40 min incubation period of [^{125}I]-labelled CRP with non-stimulated neutrophils resulted in the degradation rate of [^{125}I]-labelled CRP in the cell free medium to equal that of non-stimulated neutrophils and to be greater than that of the conditioned medium from non-stimulated neutrophils (Table 5).

Table 5. Degradation of [^{125}I]-labelled CRP by PMA-stimulated neutrophils: Influence of [^{125}I]-labelled CRP on the proteolytic activity of the extracellular medium during cellular degradation. Reactions (1 ml) contained 100 µg [^{125}I]-labelled CRP and either PMA-stimulated neutrophils ($10x10^6$) or the conditioned medium from these cells. Degradation was measured as 10% (w/v) TCA-soluble peptides and expressed as µg [^{125}I]-labelled CRP/ml. Neutrophil-mediated or conditioned medium degradation was measured at specific times (**). Degradation in the extracellular medium, obtained by removing the cells from the neutrophil-mediated reactions at various times (**) and returning the cell-free medium to 37°C, was measured at the indicated times (*).

Degraded [^{125}I]-labelled CRP (µg/ml)					
+PMA					
	Conditioned medium	Neutrophil-mediated	Extracellular medium		
Time (min)			60*	120	180
10**	3	5	8	18	25
20	4	8	16	28	39
40	7	17	27	47	56
60	10	27	-	47	56
120	12	47	-	-	56
180	16	56	-	-	-
–PMA					
10	2	3	4	5	7
20	3	4	6	8	11
40	4	6	10	13	18
60	6	10	-	13	19
120	9	13	-	-	19
180	13	18	-	-	-

4.B.2.2.1.1. Quantification of the extracellular proteolytic activity

The proteolytic activity released from neutrophils during an incubation with CRP was further demonstrated by directly assaying the enzymatic activity of the cell-free medium, following dissociation of extracellular CRP and enzyme. Dissociation of extracellular proteolytic activity and CRP could not be attained either by phosphorylethanolamine agarose affinity chromatography, known to bind CRP (Shephard *et al.*, 1988), or by gel filtration of the extracellular medium in 1M NaCl (data not shown). The extracellular proteolytic activity could, however, be fully dissociated from the ligand when more than 90% of the CRP was degraded to TCA-soluble peptides. For this a CRP-released conditioned medium was prepared by incubating unlabelled CRP (100 µg) with PMA-stimulated neutrophils ($10x10^6$) for 40 min, after which the cells were removed and the cell-

free medium returned to 37°C to continue degradation. At a time when more than 90% of the unlabelled CRP remaining in the extracellular medium was degraded (estimated to be at 20 hr from an identical reaction containing [^{125}I]-labelled CRP), [^{125}I]-labelled CRP (100 µg) was added. Degradation of this added [^{125}I]-labelled CRP by the CRP-released conditioned medium equalled that of the [^{125}I]-labelled CRP-degrading activity of PMA-stimulated neutrophils and was faster than that of control conditioned medium prepared from PMA-stimulated cells in the absence of CRP (Table 6).

Table 6. Quantification of the proteolytic activity released from PMA-stimulated neutrophils by CRP. Degradation reactions (37°C) contained 100 µg [^{125}I]-labelled CRP/ml and either $10x10^6$ PMA-stimulated neutrophils or the conditioned medium from these cells prepared in both the presence and the absence (control) of CRP (as described in the Materials and methods). Degradation, at the indicated time points, was measured as 10% (w/v) TCA-soluble peptides and expressed as µg [^{125}I]-labelled CRP/ml.

Degraded [^{125}I]-labelledCRP (µg/ml)			
Time (hr)	Neutrophil-mediated	CRP-released conditioned medium	Control conditioned medium
1	25	26	9
2	42	42	14
3	53	52	19

4.B.2.2.1.2. [^{125}I]-labelled CRP-degrading activity of cytoskeleton and membranes from neutrophils that have been incubated with CRP

Since a CRP-mediated egress of enzymatic activity from neutrophils was observed, the possibility that this occurs from both the membrane and the cytoskeleton was investigated. Non-stimulated and PMA-stimulated neutrophils were incubated with unlabelled CRP for a period of 40 min whereupon the cytoskeleton and membranes were isolated and assayed for CRP-degrading activity. The [^{125}I]-labelled CRP degrading activity of these fractions was then compared with that of the [^{125}I]-labelled CRP-degrading activity of membrane and cytoskeleton fractions isolated from control cells not pre-incubated with unlabelled CRP. A minimal change in the [^{125}I]-labelled CRP-degrading activity of neutrophil membrane and cytoskeleton fractions occurred as a result of

stimulating neutrophils with PMA in the absence of CRP (Table 7). However, when these fractions were isolated from non-stimulated and PMA-stimulated neutrophils, which had been preincubated with unlabelled CRP, a substantial decrease in the CRP-degrading activity of these fractions was observed (Table 7). This phenomenon was dependent on the concentration of CRP and the time of incubation of CRP with neutrophils. A minimum of 2 μg CRP induced this loss of proteolytic activity from the neutrophil cytoskeleton and membrane. The maximum loss of proteolytic activity occurred when 500 μg CRP was incubated for 40 min with $10x10^6$ PMA-stimulated neutrophils (Table 7).

Table 7. [^{125}I]-labelled CRP-degrading activity of cytoskeleton and membranes isolated from neutrophils that have been incubated with CRP. Reactions contained 100 μg [^{125}I]-labelled CRP/ml and either the membrane or cytoskeleton from $10x10^6$ neutrophils which had been incubated for 40 min (37°C) without or with CRP (2 μg or 500 μg) and in the absence (–) or presence (+) of PMA (10 ng/ml). Degradation, at the indicated time points, was measured as 10% (w/v) TCA-soluble peptides and expressed as μg [^{125}I]-labelled CRP/ml.

	Degraded [^{125}I]-labelled CRP (μg/ml)						
	PMA (10 ng/ml)	–	+	–	+	–	+
	CRP (μg/ml)	0	0	2	2	500	500
	Time (hr)						
Membrane	1	19	14	13	6	11	2
	3	45	43	41	11	39	7
Cytoskeleton	1	16	15	10	5	8	1
	3	38	37	32	11	27	6

4.B.2.2.2. The effect of kinase inhibitors on the rate of degradation of CRP by PMA-stimulated neutrophils

The degradation of [^{125}I]-labelled CRP by PMA-stimulated neutrophils was concentration dependently inhibited by H7 and TFP to a rate similar to that by non-stimulated neutrophils (Table 8). A minimum concentration of 20 μM and 10 μM, respectively, and a preincubation period of 5 min was required for inhibition of degradation. Increasing the concentration of H7 and TFP beyond 50 μM and 20 μM respectively and extending the preincubation period of the cells with these inhibitors to 30 min did not give rise to further inhibition (Table 8). At all concentrations of TFP and H7 used and with the use of dimethyl sulfoxide to solubilise the inhibitors, cell death was not observed. Neither leupeptin nor E-64 altered or inhibited the rate of [^{125}I]-labelled CRP degradation by PMA-stimulated neutrophils (Table 8). None of the inhibitors tested affected the rate of [^{125}I]-labelled CRP degradation by non-stimulated neutrophils (Table 8).

Table 8. Influence of inhibitors on [^{125}I]-labelled CRP degradation by neutrophils. Reactions (37°C) contained 100 μg [^{125}I]-labelled CRP/ml and $10x10^6$ non-stimulated or PMA-stimulated (10 ng/ml) neutrophils that had been pre-incubated (37°C, 15 min) with or without the indicated inhibitor. Degradation, at the indicated time points, was measured as 10% (w/v) TCA-soluble peptides and expressed as μg [^{125}I]-labelled CRP/ml. H7: 1-(5-isoquinolinesulphonyl)-2-methylpiperazine. TFP: Trifluoperazine.

		Degraded [^{125}I]-labelled CRP/ml	
	Inhibitor	Time (min)	
		30	60
Neutrophil-mediated (+PMA)	None	15	23
	H7 (10 μM)	13	18
	H7 (20 μM)	11	16
	H7 (50 μM)	6	10
	TFP (10 μM)	12	17
	TFP (20 μM)	6	10
	Leupeptin (100 μM)	16	24
	E-64 (10 μM)	15	24
Neutrophil-mediated (–PMA)	None	6	10
	H7 (50 μM)	7	11
	TFP (20 μM)	7	11
	Leupeptin (100 μM)	8	12
	E-64 (10 μM)	7	11

4.B.3. DISCUSSION

4.B.3.1. Identification of the CRP-degrading protease

The degradation of CRP observed during incubation with neutrophils, has been previously shown to be due to a neutrophil membrane-associated protease which is maximally activated by stimulation with PMA (Shephard *et al.*, 1989; Shephard *et al.*, 1990). In this study, it was shown that the neutrophil cytoskeleton fraction, prepared by non-ionic detergent extraction of the cell surface membrane proteins, also contains a neutral protease capable of degrading CRP. CRP degradation products with identical apparent molecular mass values were generated by the proteolytic activity of neutrophil cytoskeleton and membrane fractions and PMA-stimulated neutrophil conditioned medium. These products differ in molecular mass from those produced by the action of neutrophil lysosomal enzymes on CRP. Thus, the CRP-degrading protease within the neutrophil cytoskeleton fraction, the neutrophil membrane fraction and PMA-stimulated conditioned medium could be the same but distinct from the CRP-degrading proteases within the neutrophil granules. This is supported by the observation that the enzyme within each of these three neutrophil fractions could be dissociated by SDS-PAGE into four discrete bands which migrated to positions with identical electrophoretic mobility at the apparent molecular mass values of 209 kDa, 316 kDa, 398 kDa and 501 kDa. The association of this high molecular mass neutral protease with both the neutrophil membrane and cytoskeleton fraction suggests that it may be a sub-membrane protein localised at sites of interaction of the cytoskeleton with the membrane. Since actin is a component of the cytoskeleton fraction (Yassim *et al.*, 1985) it is possible that the CRP-degrading protease is a sub-membrane protease which interacts with actin and lipids at the cytoplasmic surface of the plasma membrane and may, therefore, be localised at the interface of the cytoskeleton with the membrane.

The CRP-degrading neutrophil protease described here appears to be different from a previously described serine protease, also associated with the neutrophil cytoskeleton and membrane and reported to have an apparent molecular mass of 300 kDa (Pontremoli *et al.*, 1986). This 300 kDa protease was not demonstrated to display any subunit or aggregation characteristics and was shown to be almost completely released into the extracellular medium when the cells were stimulated with PMA, via the activation of PKC.

4.B.3.2. Mechanism of CRP proteolysis

The results indicate that the mechanism for neutrophil-mediated CRP degradation resides in the ability of CRP to mediate a PMA-upregulatable egress of proteolytic activity from both the membrane and the cytoskeleton, which promotes its degradation in the extracellular milieu. Although some cleavage of CRP could occur at the neutrophil membrane during interaction of CRP with the cell, extracellular degradation of CRP also occurs through the released protease interacting with CRP in the extracellular medium where they form a tight complex that is not easily dissociated. Extracellular degradation of CRP would allow the generation of CRP degradation products which may then associate with the neutrophil surface as suggested by Shephard *et al.* (1989).

The CRP-mediated egress of proteolytic activity was complete within 40 min which may relate to the time required to saturate the neutrophil surface with CRP. Perturbation of the neutrophil membrane, in the region of attachment of the membrane to the cytoskeleton, as a result of CRP binding to the putative CRP receptor (Shephard *et al.*, 1989; Buchta *et al.*, 1987a; Kilpatrick and Volanakis, 1985) could account for the observed egress of proteolytic activity from the neutrophil. Results from the previous section suggest the putative CRP receptor to be close to the CD11c/CD18 receptor on neutrophils.

The PMA-upregulatable, CRP-mediated release of CRP-degrading activity from the neutrophil, appears to involve the action of cellular kinases. Since no change in the rate of CRP degradation was achieved by sustaining PKC in the membrane, by inhibiting calpain with the thiol protease inhibitors leupeptin and E-64, the action of PKC may not be responsible for enhancing the CRP-mediated egress of proteolytic activity from the neutrophil. On the other hand H7 and TFP, which are known to inhibit not only effector dependent PKC, but also the cyclic nucleotide-dependent kinases and calmodulin dependent processes activated by PMA (Hidaka *et al.*, 1984; Seifert and Schachtele, 1988), were shown to be inhibitory for the rate of degradation of CRP by neutrophils. Thus, neutrophil kinase dependent processes other than PKC may be involved in the enhancement of the PMA-upregulatable, CRP-mediated egress of CRP-degrading activity from neutrophil membranes and cytoskeleton. The kinase(s) responsible may be suggested to achieve this enhancement of proteolytic egress from PMA-stimulated neutrophils by the phosphorylation of a specific intracellular substrate.

CHAPTER 5

INTERACTION OF FIBRINOGEN WITH NEUTROPHILS

RESULTS AND DISCUSSION

LIST OF CONTENTS

5.A. PROTEOLYSIS OF FIBRINOGEN BY A NEUTROPHIL MEMBRANE-ASSOCIATED PROTEASE

5.A.1. INTRODUCTION

Human fbg can be degraded by thrombin (Bailey *et al.*, 1951), plasmin (Pizzo *et al.*, 1972), neutrophil lysates (Bilezekian and Nossel, 1977; Gramse *et al.*, 1977) and the human neutrophil proteases, elastase (HNE, Weitz *et al.*, 1986) and cathepsin G (Gramse *et al.*, 1980), to yield distinct products. Thrombin cleaves the Aα and Bβ chains at positions 16 and 17 and positions 14 and 15 from their N-termini respectively (Fig 2), resulting in the generation of fibrinopeptides A and B (FPA and FPB) and, following a subsequent series of reactions, fibrin is generated (Bailey *et al.*, 1951). Plasmin cleaves fbg initially from the C-terminus of the Aα chain followed by the N-terminus of the Bβ chain and finally the γ chain, which is fairly resistant to degradation and is only cleaved once large parts of the Aα and the Bβ chain have been split off (Pizzo *et al.*, 1972; Ferguson *et al.*, 1975; Azpiazu and Chapman, 1992). A consequence of this cleavage of fbg by plasmin is the generation of specific products (fragments A-E, Fig 2) of which fragments D and E possess anticoagulant activity (Doolittle, 1984; Olexa *et al.*, 1981; Larrieu *et al.*, 1972). Neutrophil fibrinogenolysis is characterised by N-terminal cleavage of all three constituent fbg chains to yield FPA- and FPB-containing peptides which display antigenic characteristics of D and E and also demonstrate anticoagulant activity (Gramse *et al.*, 1977; Gramse *et al.*, 1980; Plow and Edgington, 1975). In addition, neutrophil generated fragments that are larger than the plasmin derived fragment X (Fig 2) are found to be non-clottable by thrombin (Plow and Edgington, 1975). A characteristic of fbg degradation by neutrophil lysates (Bilezekian and Nossel, 1977) and HNE (Weitz *et al.*, 1986; Wright *et al.*, 1988; Weitz *et al.*, 1987) is the generation of an Aα1-21 peptide through the cleavage of the bond between the amino acids Val and Glu at positions 21 and 22, respectively, from the N-terminus of the Aα chain. Plasma levels of this peptide have been used as a marker of HNE activity *in vivo* and are proposed to be useful for monitoring HNE activity in conditions where α_1AT inhibitor activity is compromised and in subjects with homozygous and heterogeneous deficiency of this enzyme inhibitor (Weitz *et al.*, 1986; Weitz *et al.*, 1992). This study endeavours to characterise the degradation of fbg by the neutrophil membrane-associated protease, previously shown to degrade CRP (Shephard *et al.*, 1989; Shephard *et al.*, 1990), and evaluates the capacity of this protease to act as an alternative pathway to fibrinogenolysis by plasmin and neutrophil lysosomal enzymes. The nature and function of the resultant fbg degradation products will also be assessed.

5.A.2. RESULTS

5.A.2.1. Comparison of fibrinogen degradation by neutrophil conditioned medium, neutrophil cytoskeleton, neutrophil membranes, neutrophil lysosomal enzymes and plasmin

When PMA-stimulated neutrophil conditioned medium was incubated with [^{125}I]-labelled fbg and analysed by SDS-PAGE (5-20% non-reduced) at various time intervals, a progressive decrease in the size of the intact fbg molecule (apparent molecular mass 340 kDa) and the generation of [^{125}I]-labelled fbg derivatives with apparent molecular mass values less than 340 kDa was observed (Fig 9A). The size of the degradation products, formed at a particular time point, was dependent on the concentration of enzyme, related to the number of neutrophils from which the conditioned medium was prepared (Fig 9A and 10B). The neutrophil cytoskeleton and membrane fractions generated [^{125}I]-labelled degradation products of identical molecular size to those produced by the neutrophil conditioned medium (Fig 9A and Fig 10) and degradation was similarly shown to be dependent on time and the concentration of the enzyme source (data not shown). Each of the three neutrophil fractions generated terminal [^{125}I]-labelled fbg degradation products which migrated on SDS-PAGE (5-20% non-reduced) to positions corresponding to apparent molecular mass values of 270 kDa, 200 kDa, 100 kDa and less than 40 kDa (Fig 9A and 10). The terminal fbg products generated by these neutrophil enzyme sources differed in molecular mass to those generated at 20 hr by plasmin, in the presence and absence of $CaCl_2$, neutrophil lysosomal enzymes and pure HNE (Fig 10). The terminal fbg degradation products generated by neutrophil conditioned medium, did not alter in apparent molecular mass when the Ca^{2+} was chelated with EGTA (Fig 11). The 100 kDa product, generated by the neutrophil conditioned medium, was found by two-dimensional iso-electrophoresis to have a pI of 6.25-6.3 which was similar to the pI of 6.15-6.35 found for the plasmin generated 90 kDa product thought to be fragment D (Fig 12). The high molecular mass fbg terminal products (270 kDa, 200 kDa and 100 kDa) generated in the presence of PMA-stimulated neutrophil conditioned medium were shown to be plasmin sensitive (Fig 13).

In addition to the generation of high molecular mass fbg products, the three neutrophil fractions also generated TCA-soluble peptides. The generation of these peptides was dependent upon the time of incubation of the [^{125}I]-labelled fbg with the specific enzyme source as well as the concentration of enzyme (Table 9).

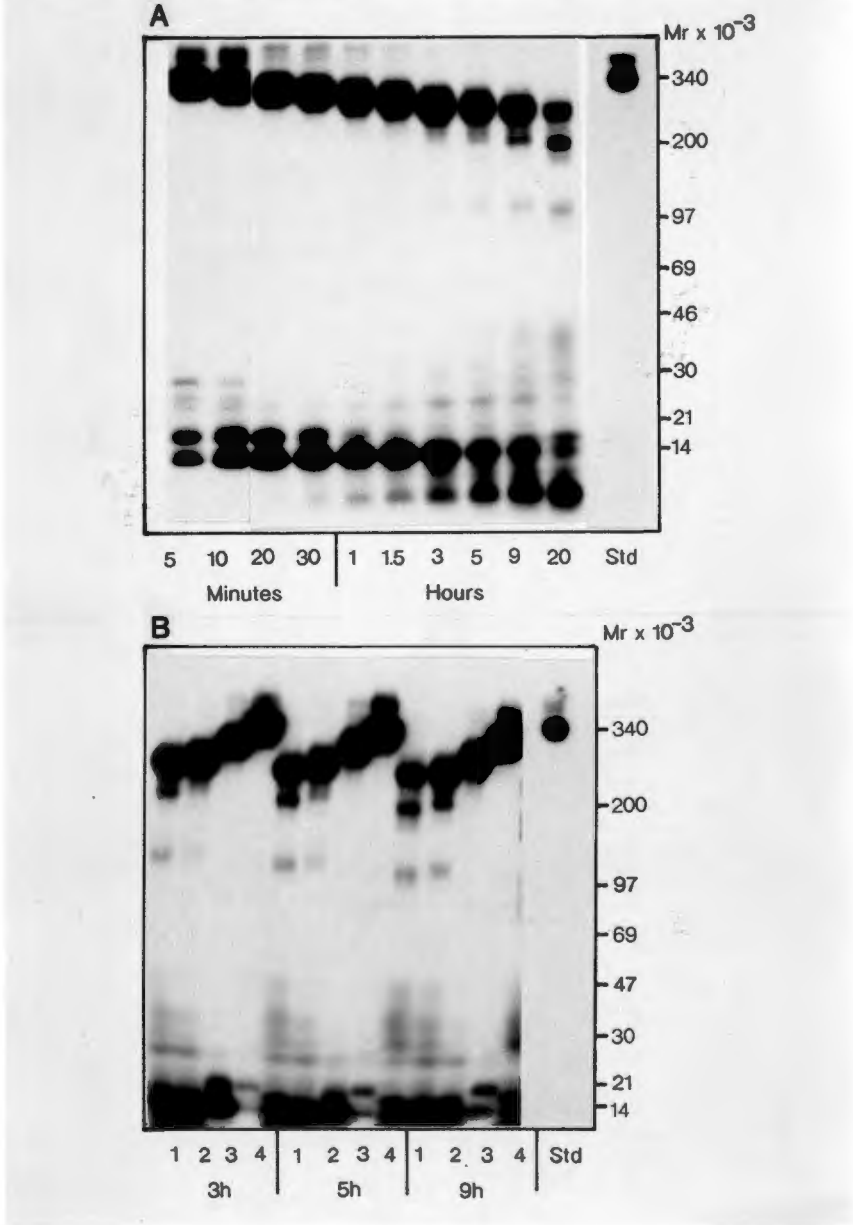

Figure 9. The nature of the [^{125}I]-labelled fibrinogen degradation products generated by neutrophil conditioned medium. [^{125}I]-labelled fbg (2 mg) was incubated with the conditioned medium from PMA-stimulated neutrophils and, at the indicated times, aliquots of the reaction mixture were subjected to SDS-PAGE (5-20% non-reduced) and autoradiographic analysis, as descibed in the Materials and methods. **A**: Nature of the [^{125}I]-labelled fbg degradation products generated by the conditioned medium from 5x10^6 PMA-stimulated neutrophils occurring over time. **B**: Nature of the [^{125}I]-labelled fbg degradation products generated when the conditioned medium was prepared from 10x10^6 PMA-stimulated neutrophils (track 1), 5x10^6 PMA-stimulated neutrophils (track 2), 2.5x10^6 PMA-stimulated neutrophils (track 3) and 1x10^6 PMA-stimulated neutrophils (track 4). Undigested [^{125}I]-labelled fbg is shown in the track marked Std.

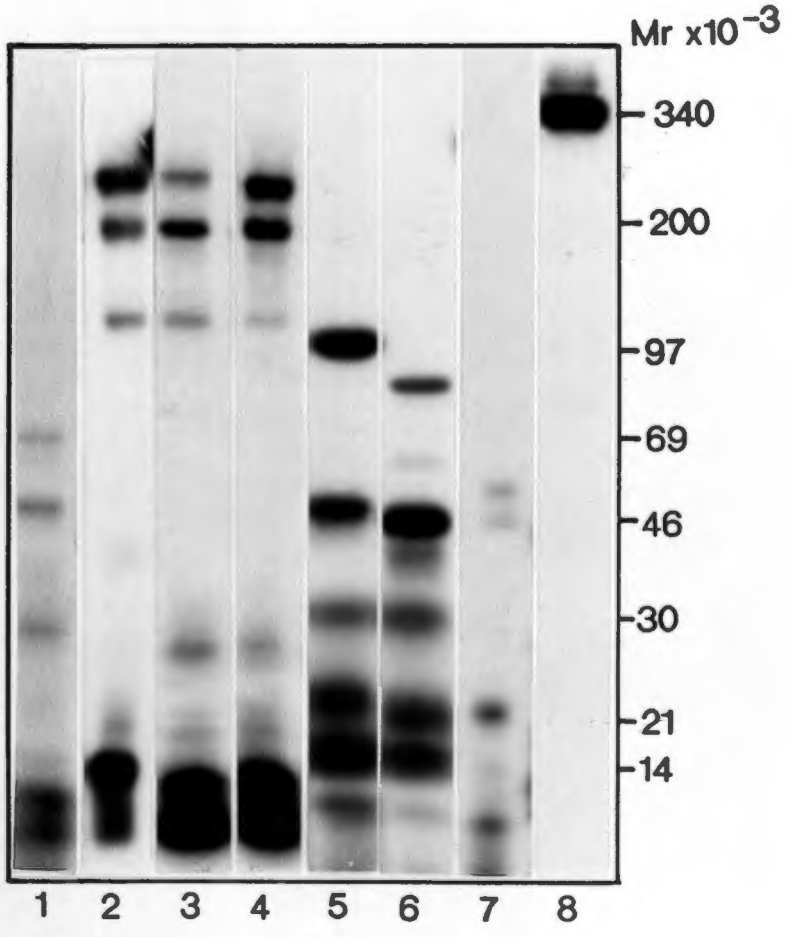

Figure 10. Degradation of [^{125}I]-labelled fibrinogen. Degradation reactions (37^{o}C) containing [^{125}I]-labelled fbg (2 mg) and the enzyme source, in a final volume of 1 ml, were prepared and the nature of the products, generated at 20 hr, was assessed by SDS-PAGE (5-20% non-reduced), as described in the Materials and Methods section. Products were generated by HNE (2 μg, track 1); conditioned medium from 5x10^{6} PMA-stimulated neutrophils (track 2), neutrophil cytoskeletons (25 μg, track 3), neutrophil membranes (25 μg, track 4), urokinase-activated porcine plasminogen in the presence of $CaCl_2$ (2 mM, track 5) or EGTA (10 mM, track 6) and lysosomal enzymes released from neutrophils by FMLP (1 μM) in the presence of cytochalasin B (5 μg/ml, track 7). Track 8 shows undigested [^{125}I]-labelled fbg.

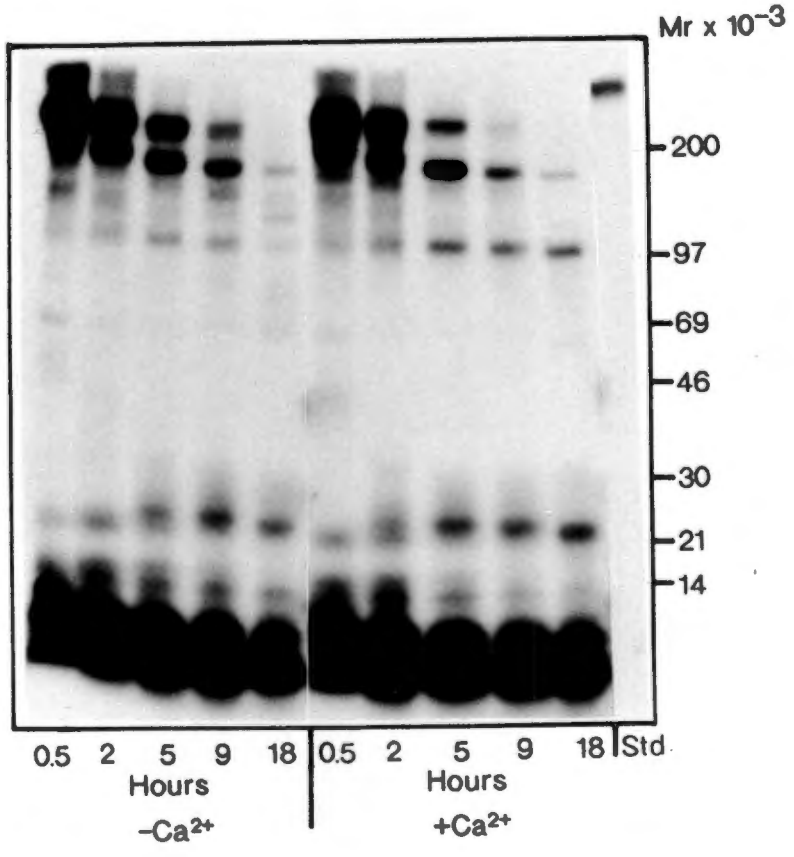

Figure 11. Influence of Ca^{2+} on the degradation of [^{125}I]-labelled fibrinogen by neutrophil conditioned medium. [^{125}I]-labelled fbg (2 mg) was incubated (37°C) with the conditioned medium from $5x10^6$ PMA-stimulated neutrophils in the presence or absence of EGTA (10 mM) in a final volume of 1 ml. At the indicated times aliquots of the reactions were subject to analysis by SDS-PAGE (5-20% non-reduced) as described in the Materials and methods section. Undigested [^{125}I]-labelled fbg is indicated in the track marked Std.

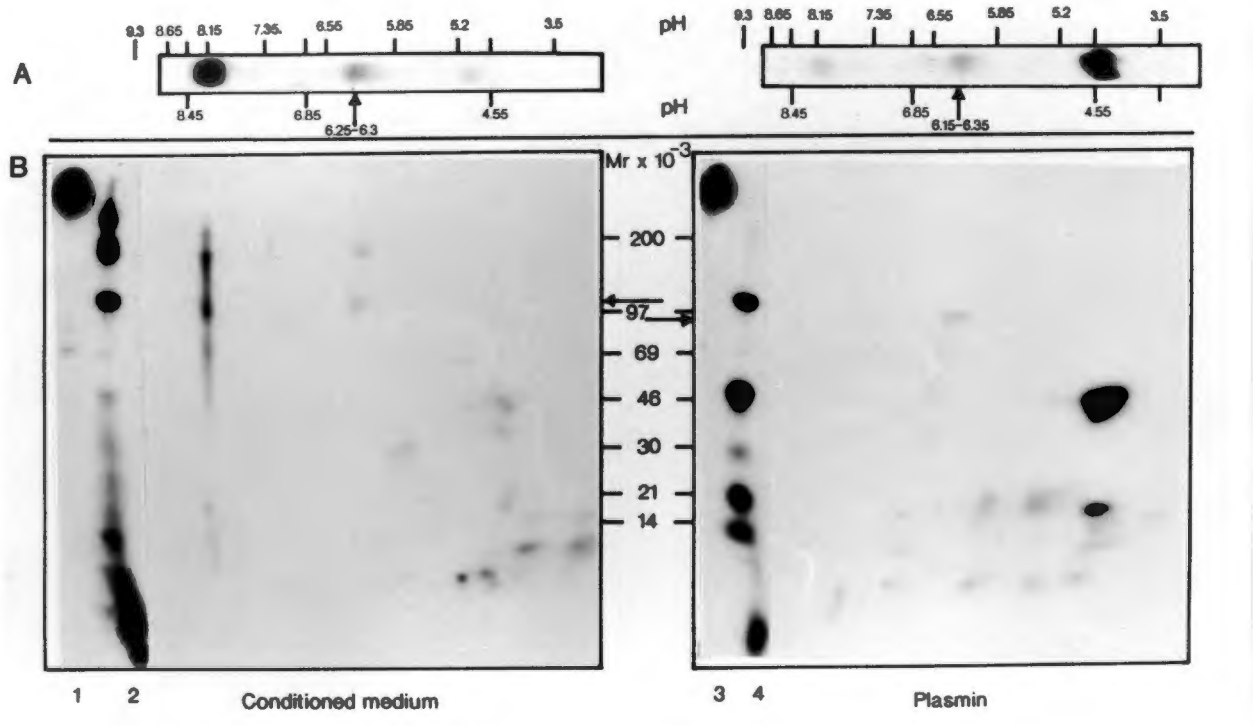

Figure 12. Iso-electric focusing of plasmin and neutrophil conditioned medium generated [^{125}I]-labelled fibrinogen degradation products, followed by two dimensional SDS-PAGE. A. Terminal [^{125}I]-labelled fbg degradation products of a plasmin as well as a neutrophil conditioned medium degradation reaction (as indicated) were analysed on an isoelectric PAG plate with a broad pH range between 3.5 and 9.5 (as described in the Materials and methods). Broad range pH markers (3.5-9.3) are indicated. **B.** The terminal degradation products separated on the isoelectric PAG plates (A) were then subjected to two dimensional SDS-PAGE (5-20% non-reduced) followed by autoradiography. The terminal degradation products of apparent molecular mass 90 kDa (generated by plasmin degradation) and 100 kDa (generated by the neutrophil conditioned medium) and their corresponding isoelectric points are indicated with arrows. Tracks 1 and 3 represent undigested [^{125}I]-labelled fbg. Track 2 represents the terminal [^{125}I]-labelled fbg degradation products generated by neutrophil conditioned medium in the first dimension and track 4 shows the terminal [^{125}I]-labelled fbg degradation products generated by plasmin.

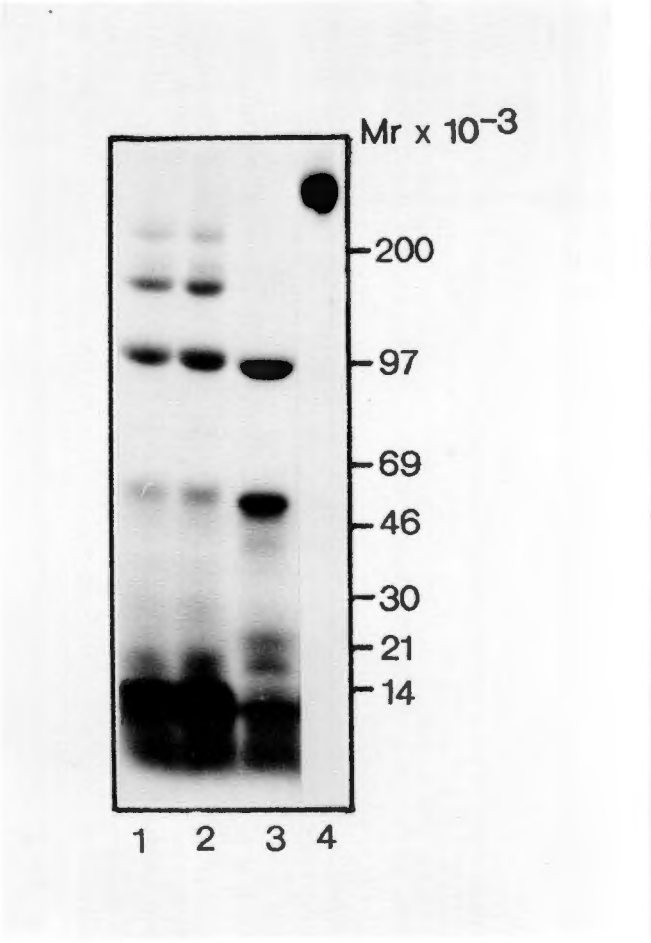

Figure 13. Degradation of [^{125}I]-labelled fibrinogen by neutrophil conditioned medium, followed by plasmin. [^{125}I]-labelled fbg (2 mg) was incubated (37°C, 18 hr) with the conditioned medium from $5x10^6$ neutrophils and the fbg products generated were analysed by SDS-PAGE (5-20% non reduced) followed by autoradiography as described in the Materials and methods section (track 1). The reaction mix was subjected to a further 30 min incubation (37°C) without (track 2) or with (track 3) urokinase-activated plasminogen in the presence of $CaCl_2$ (2 mM). Undigested [^{125}I]-labelled fbg is indicated in track 4.

Table 9. Degradation of [^{125}I]-labelled fibrinogen. Degradation reactions (37°C) contained [^{125}I]-labelled fbg (2 mg) and either conditioned medium (from 2.5x10^6, 5x10^6 or 1x10^7 PMA-stimulated neutrophils), isolated neutrophil cytoskeletons (12.5, 25 or 50 µg of protein) or isolated neutrophil membranes (12.5, 25 or 50 µg of protein) in a final volume of 1 ml (as described in Materials and methods). Degraded fbg, measured as 10% (w/v) TCA-soluble peptide formation, at the indicated time, is expressed as µg [^{125}I]-labelled fbg products/ml.

Degraded [^{125}I]-labelled fibrinogen (µg/ml)									
Time	Conditioned medium (neutrophils/ml)			Cytoskeleton protein (µg/ml)			Membrane Protein (µg/ml)		
	2.5x10^6	5x10^6	10x10^6	12.5	25	50	12.5	25	50
5 min	20	40	80	40	120	200	60	100	160
20 min	60	140	200	120	260	420	140	240	420
1 hr	160	240	440	200	440	640	220	400	660
3 hr	380	560	640	280	660	820	360	660	800
9 hr	660	820	880	560	920	960	640	810	940
20 hr	810	920	940	720	960	980	770	860	1000

No inhibition of [^{125}I]-labelled fbg (1 mg) degradation by neutrophil conditioned medium (from 5x10^6 cells) was achieved with ε-aminocaproic acid (200 mM), aprotinin (100 µM), ethylene diamine tetra acetic acid (EDTA, 10 mM, metalloprotease inhibitor), leupeptin (100 µM, thiol protease inhibitor), phosphoramidon (10 µM, metalloprotease inhibitor), TLCK (5 mM, trypsin inhibitor), ATEE (50 mM, trypsin inhibitor), the elastase inhibitors Suc-(Ala)$_3$CH$_2$Cl (1mM), Suc-(Ala)$_2$-ValCH$_2$Cl (1mM), or the cathepsin G inhibitor Suc-(Ala)$_2$-Pro-PheCH$_2$Cl (1mM) (Table 10). Increasing the concentrations of these inhibitors 100 fold did not change the results. On the other hand, MeO-Suc-(Ala)$_2$-Pro-ValCH$_2$Cl (1 mM), an inhibitor of proteases with specificities related to alanine or valine bonds, and α_1AT (1.5 mg/ml, elastase inhibitor) reduced the production of TCA-soluble [^{125}I]-labelled fbg peptide formation, by neutrophil conditioned medium, by 50% and 80% respectively, at 20 hr (Table 10). The formation of [^{125}I]-labelled TCA-soluble fbg peptides at 20 hr was inhibited by greater than 95% by the serine protease inhibitor PMSF (1mM) and by 98% by the serine protease inhibitor analogue, AEBSF (1mM) (Table 10). Identical results were obtained when the [^{125}I]-labelled fbg was degraded with either neutrophil cytoskeleton or neutrophil membrane fractions (data not shown).

Table 10. Influence of inhibitors on the degradation of [^{125}I]-labelled fibrinogen by neutrophil conditioned medium. PMA-stimulated neutrophil conditioned medium from $5x10^6$ neutrophils was preincubated (37°C, 15 min) with various enzyme inhibitors whereafter the conditioned medium was incubated (37°C, 20 hr) with [^{125}I]-labelled fbg (2 mg). Degradation was measured as 10% (w/v) TCA-soluble peptides and was expressed as µg degraded [^{125}I]-labelled fbg generated per ml.

Degraded [^{126}I]-labelled fibrinogen (µg/ml)		
Inhibitor	Concentration	Time 20 hr
None		460
$MeOSuc(Ala)_2ProValCH_2Cl$	1 mM	230
PMSF	1 mM	23
α_1-antitrypsin	1.5 mg/ml	92
AEBSF	1 mM	9

5.A.2.2. Molecular size of the fibrinogen degrading protease

Neutrophil membranes (200 µg), neutrophil cytoskeleton (200 µg) and PMA-stimulated neutrophil conditioned medium (from $50x10^6$ cells) were subjected to SDS-PAGE (3-13% non-reduced). Gel slices of 2 mm thickness were then incubated with [^{125}I]-labelled fbg (1mg) and TCA-soluble peptide formation was monitored. Fibrinogenolytic activity of all three neutrophil fractions was recovered from gel slices corresponding to apparent molecular mass values of 501 kDa, 398 kDa, 316 kDa and 209 kDa. Of the total proteolytic activity loaded, 13%, 40%, 30% and 17% was recovered from these respective bands, following a 24 hr incubation of each gel slice with [^{125}I]-labelled fbg. The bands at apparent molecular mass values 501 kDa, 398 kDa, 316 kDa and 209 kDa thus generated approximately 180 µg, 500 µg, 300 µg and 230 µg TCA-soluble [^{125}I]-labelled fbg peptides/ml, respectively, irrespective of the enzyme source.

5.A.2.3. Functional assessment of the fibrinogen degradation products

When thrombin is added to fbg, clotting is induced. The time taken for a clot to form is referred to as the thrombin clotting time and is dependent on the specific integrity of the fbg molecule (Gramse *et al.*, 1977; Plow and Edgington, 1975). Clot formation of the intact [^{125}I]-labelled fbg molecule (340 kDa), following the addition of thrombin, was observed within 15 seconds while a notable, progressive increase in the thrombin clotting time was observed when the [^{125}I]-labelled fbg was degraded by neutrophil conditioned medium (Table 11). A fbg product with an apparent molecular mass of 330 kDa (30 min, Fig 9A), prolonged the thrombin induced clotting time to 25

sec (Table 11). Further reduction of the fbg molecule to an apparent molecular mass of 310 kDa (1.5 hr, Fig 9A) caused the clotting time to be greater than 3 min (Table 11). When the fbg molecule was reduced to an apparent molecular mass of 290 kDa (3hr, Fig 9A) no clot formation was observed following treatment with thrombin (Table 11). A similar prolongation of the thrombin clotting time was achieved when [^{125}I]-labelled fbg was degraded by either neutrophil cytoskeleton or membrane fractions. Generation of fragment X (±240 kDa) by plasmin (Fig 14) prolonged the thrombin induced clotting time beyond 25 sec (Table 11).

None of the [^{125}I]-labelled fbg products, generated by either neutrophil conditioned medium, isolated cytokeletons or membranes, exhibited any anticoagulant activity. In contrast, the [^{125}I]-labelled fbg degradation products generated by plasmin, namely fragments Y, D and E, all exhibited anticoagulant activity and prolonged the thrombin-induced clotting activity of intact fbg beyond 3 min (Table 11).

Table 11. The thrombin clotting time of fibrinogen and fibrinogen degradation products of varying molecular mass. Fbg degradation products were generated by either PMA-stimulated neutrophil conditioned medium or plasmin, as described in the Materials and methods. Thrombin (2 i.u./10 µl) was added to intact fbg and the fbg products and the thrombin clotting time was measured visually and expressed in seconds.

Molecular mass of fibrinogen/fibrinogen products (kDa)			
Intact fibrinogen	Conditioned medium degradation	Plasmin degradation	Thrombin clotting time (sec)
340	-	-	15
-	330	-	25
-	310	-	>180
-	290	-	NO CLOT
-	-	±240 (X)	>25
-	-	155 (Y)	>180
-	-	90 (D)	>180
-	-	41 (E)	>180

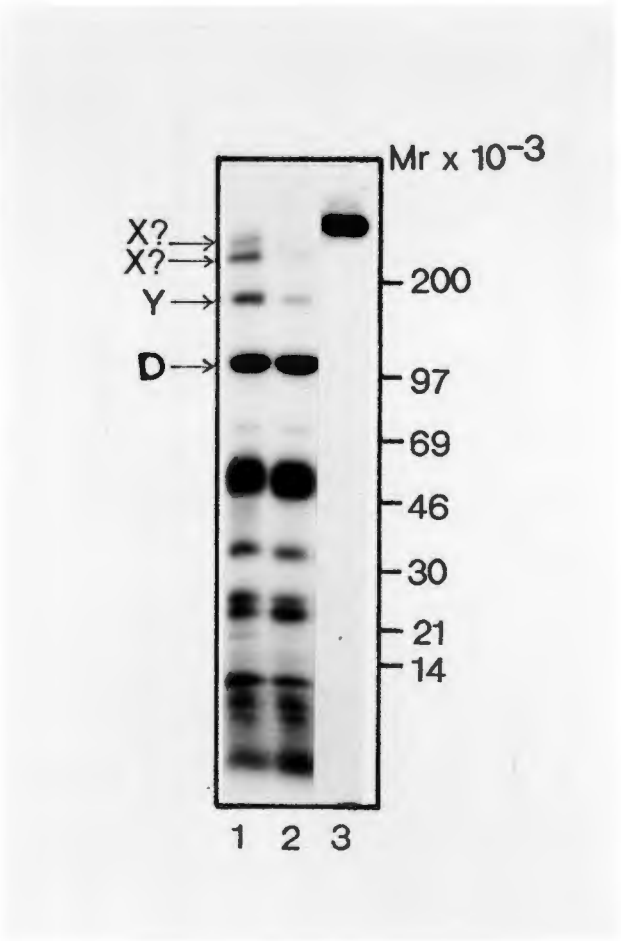

Figure 14. The nature of the [^{125}I]-labelled fibrinogen degradation products generated by plasmin. [^{125}I]-labelled fbg (1 mg) was incubated (37°C, 30 min) with urokinase-activated plasminogen (0.5 μg) in the presence of $CaCl_2$ (2 mM, track 1) or EGTA (10 mM, track 2), in a final volume of 1 ml. The nature of the [^{125}I]-labelled fbg products were assessed by SDS-PAGE (5-20% non reduced) followed by autoradiography as described in the Materials and methods section. Fragments X, Y and D are indicated. Due to the transient nature of fragment X, two bands are indicated to be possible representatives of this product. Undigested [^{125}I]-labelled fbg is indicated in track 3.

Clot formation, following thrombin treatment, occurs through covalent crosslinks between the γ chains to generate γ-γ dimers (from Mosesson, 1992). These γ-γ dimers migrate on SDS-PAGE (10% reduced) to a position corresponding to the apparent molecular mass of 90 kDa (Fig 15). Degradation of the [^{125}I]-labelled fbg molecule, by PMA-stimulated neutrophil conditioned medium, caused a progressive decrease in the generation of these γ-γ dimers, subsequent to the addition of thrombin, as observed by the gradual decrease in the radiolabelled band migrating to an apparent molecular mass of 90 kDa (10% reduced SDS-PAGE) as degradation proceeded (Fig 15). [^{125}I]-labelled fbg products of apparent molecular mass 310 kDa, and larger (1.5 hr, Fig 9A) clearly formed γ-γ dimers (Fig 15). However, γ-γ dimer formation did not occur when fbg products with apparent molecular mass values of 290 kDa (3 hr, Fig 9A) were generated (Fig 15).

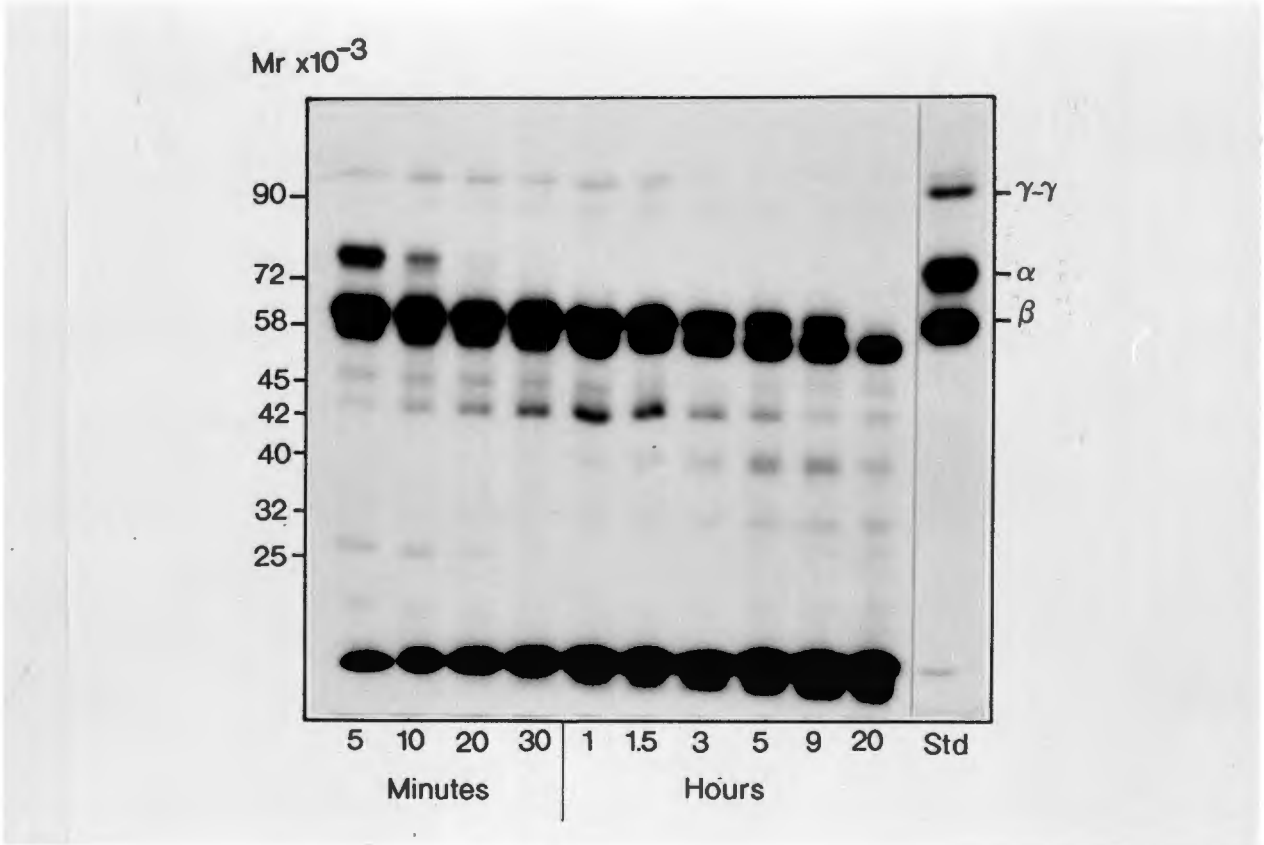

Figure 15. The ability of [^{125}I]-labelled fibrinogen degradation products to form γ-γ crosslinks. [^{125}I]-labelled fbg (1 mg) was incubated (37°C) with the conditioned medium from 5x10^{6} PMA-stimulated neutrophils, in a final volume of 1 ml. At the indicated time points, the cells were removed and thrombin (1 i.u./10 µl) was added for 3 min at 37°C. γ-γ crosslink formation was assessed by SDS-PAGE (10% reduced) followed by autoradiography, as described in the Materials and methods section. The formation of the γ-γ dimer at apparent molecular mass 90 kDa is indicated and γ-γ crosslink formation of undigested [^{125}I]-labelled fbg is shown in the track marked Std.

A similar inability to form γ-γ dimers was found following the degradation of [^{125}I]-labelled fbg to a product of apparent molecular mass 290 kDa by neutrophil cytoskeleton and neutrophil membrane fractions (not shown). When [^{125}I]-labelled fbg was digested with plasmin for 30 min, fragment X (±240 kDa) (Fig 14) was generated and visible fibrin clots with γ-γ dimer formation were produced on treatment with thrombin (not shown).

5.A.2.4. Structure of the neutrophil-derived [^{125}I]-labelled fibrinogen degradation products

In order to determine the kinetics of degradation of the constituent chains of [^{125}I]-labelled fbg by neutrophil conditioned medium as well as the size of these chains at various stages during degradation, the products were subjected to SDS-PAGE (10% reduced) followed by PAS staining and autoradiography (Fig 16A, B and C). Clarification of the kinetics was achieved by performing the autoradiography over both a 30 min and a 2.5 hr period (Fig 16A and B). Following a 5 min digestion period the intensity of the radiolabel associated with the Aα chain, migrating to an apparent molecular mass of 72 kDa, was already markedly decreased (Fig 16A and B). This degradation period coincided with the generation of radiolabelled bands migrating to positions at apparent molecular mass values of 45 kDa, 38 kDa, 28 kDa, 25 kDa and small, unresolved peptides at the front of the gel (Fig 16A and B). By 20 min no intact Aα chain (72 kDa) was detected. At 1 hr an additional band migrating to an apparent molecular mass of 42 kDa was noted and at 3 hr bands at apparent molecular mass values of 40 kDa and 32 kDa were also generated (Fig 16A and B). The radiolabel associated with these three bands was then noted to decrease as degradation proceeded to 9 hr (Fig 16A and B). As the degradation period proceeded to 9 hr there was also a gradual decrease in intensity of the bands at apparent molecular mass values 72 kDa (Aα chain, completely degraded by 20 min), 58 kDa (Bβ chain), 45 kDa, 28 kDa and 25 kDa. At the same time an initial (see 10 min time point) increase in the intensity of the radiolabel associated with the band at apparent molecular mass 58 kDa (Bβ chain) was observed followed by the gradual decrease in intensity observed as degradation proceeded to 9 hr (Fig 16A and B). A gradual increase in the intensity of the radiolabel associated with the band at apparent molecular mass of 48 kDa (γ chain) was noted as degradation proceeded to 9 hr and the intensity of the radiolabel of this band was only noted to decrease as degradation continued beyond 9 hr to 20 hr (Fig 16A and B). At the end point of digestion (20 hr) the radiolabelled bands which were still visible were those migrating to apparent molecular mass values 48 kDa, 45 kDa, 42 kDa, 40 kDa, 38 kDa, 32 kDa and less than 25 kDa (Fig 16A and B). No intact Bβ chain was detected at 20 hr. The intensity of the radiolabel associated with the band migrating to an apparent molecular mass of 48 kDa, at 20 hr, was greater than that of the γ chain of undigested fbg (Fig 16A and B). This would seem to indicate the migration of a product from either the Aα or Bβ chain, or both, to an apparent molecular mass of 48 kDa.

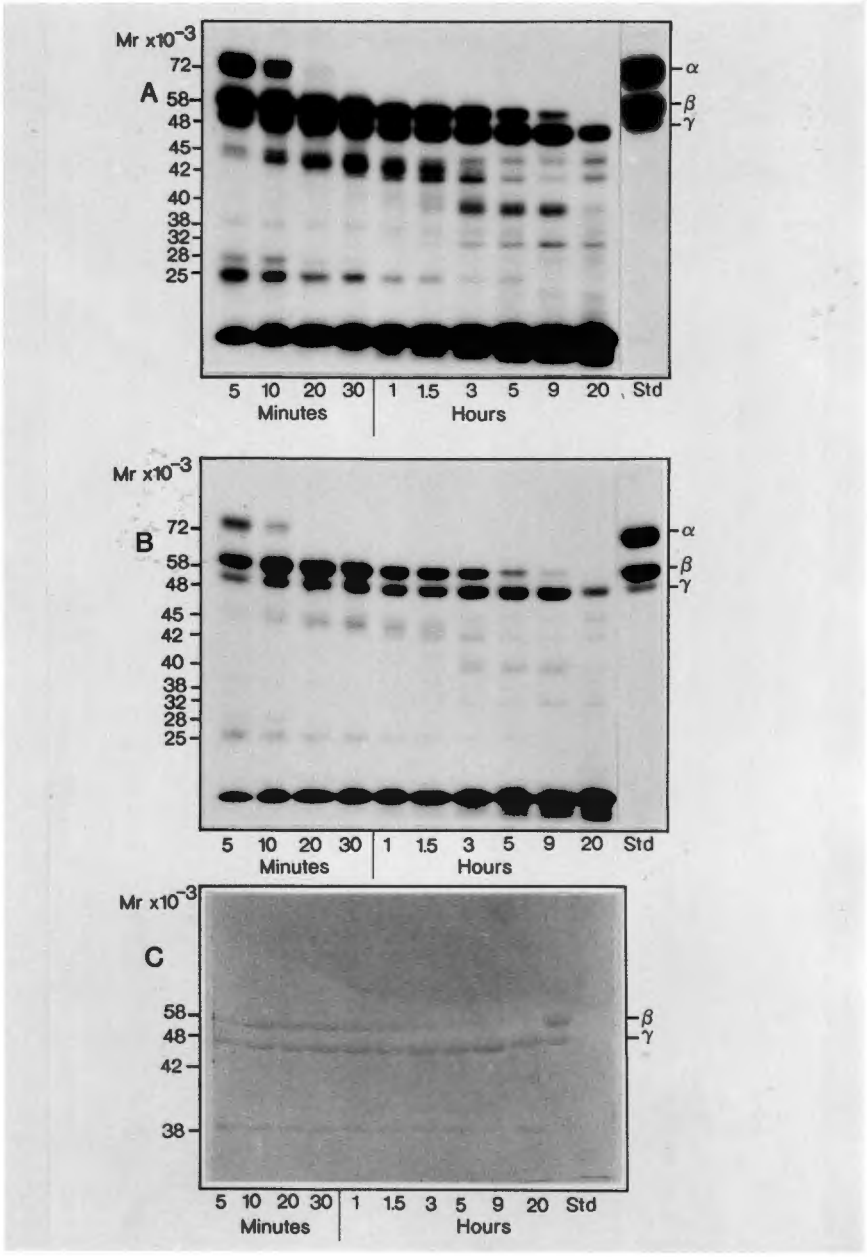

Figure 16. Structure of the [^{125}I]-labelled fibrinogen degradation products. The [^{125}I]-labelled fbg degradation products, generated at various times (5 min to 20 hr) from [^{125}I]-labelled fbg (2 mg) by the conditioned medium from $5x10^6$ PMA-stimulated neutrophils, as described in the Materials and methods, were subjected to SDS-PAGE (10% reduced) analysis. **A**: 2.5 hr autoradiographic exposure; **B**: 30 min autoradiographic exposure; **C**: products stained for carbohydrate using PAS.

Prior to digestion only the Bβ (58 kDa) and the γ chains (48 kDa) stained with PAS (Fig 16C). During the digestion period from 5 min to 9 hr those bands with apparent molecular mass values of 58 kDa, 48 kDa and 38 kDa appeared to contain carbohydrate as they were also stained with PAS (Fig 16C). The intensity of the PAS staining, associated with the band migrating to the apparent molecular mass 48 kDa (γ chain), appeared to increase as degradation proceeded to 9 hr (Fig 16C). The 42 kDa product generated at 20 hr also contained carbohydrate (Fig 16C).

The presence of the inhibitors α_1AT (1.5 mg/ml) or MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (1 mM) in these reactions inhibited degradation of the [^{125}I]-labelled fbg Bβ and the γ chains by the neutrophil conditioned medium. Degradation of the Aα chain still occurred in the presence of MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl and occurred more slowly in the presence of α_1AT (Fig 17).

Identical results, to those described above, were found if the enzyme source was from either the neutrophil cytoskeleton or membrane fractions (not shown).

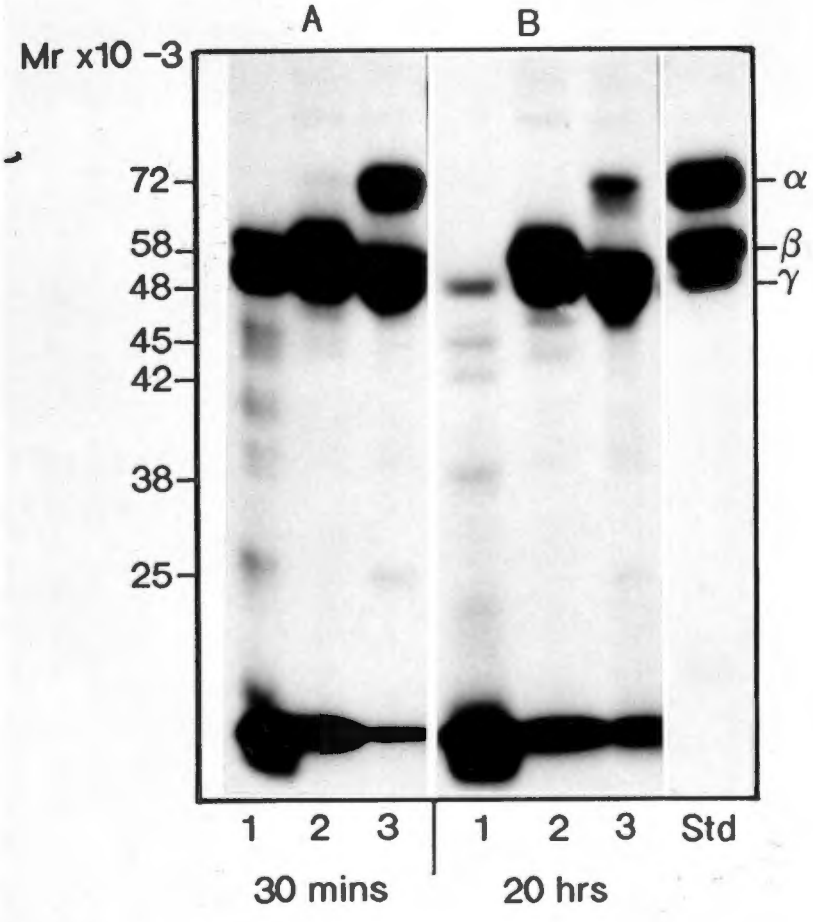

Figure 17. Effect of inhibitors on [^{125}I]-labelled fibrinogen degradation by neutrophil conditioned medium. [^{125}I]-labelled fbg (2 mg) was incubated (37°C) with conditioned medium from 5x10^6 PMA-stimulated neutrophils, in the presence or absence of either MeO-Suc-(Ala)$_2$-Pro-ValCH$_2$Cl (1 mM) or α_1AT (1.5 mg/ml). At 30 min and 20 hr the nature of the fbg degradation products was analysed by SDS-PAGE (10% reduced). **A**: nature of the products at 30 min, **B**: nature of the products at 20 hr. Track 1: products generated in the absence of inhibitor, Track 2: products generated in the presence of MeO-Suc-(Ala)$_2$-Pro-ValCH$_2$Cl (1 mM), Track 3: products generated in the presence of α_1AT (1.5 mg/ml). Undigested [^{125}I]-labelled fbg is indicated in the track marked Std.

5.A.2.5. H.p.l.c. analysis of the trichloracetic acid-soluble fibrinogen degradation products

H.p.l.c. analysis of the TCA-soluble peptides, formed from a 20 hr digestion of fbg by PMA-stimulated neutrophil conditioned medium, was performed in order to identify N-terminal or C-terminal Aα and Bβ and γ chain released peptides (Fig 18). C-terminal γ chain peptides were sought as the peptides produced by the action of the neutrophil conditioned medium were shown to lack anticoagulant activity. Based on the known elution time of synthetic peptides corresponding to the amino acid sequence at positions 397-411, 400-411 and 402-411 from the N-terminus of the γ chain, peptides eluting between 23 and 27 min were identified. A peptide eluting at 25 min was found to have an amino acid composition corresponding to the amino acid sequence from position 395-411 from the N-terminus of the γ chain (Table 12) (Fig 18A). This peptide was observed after a 5 min incubation of fbg with the neutrophil conditioned medium and reached a peak following a 3 hr incubation period.

Table 12. Amino acid analysis of the peptide with an elution time of 25 min. Gly-Glu-Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val. This corresponds to residues 395-411 from the N-terminus of the γ chain of fbg.

Amino acid	Residues found	Theoretical value
Asp	1.2	1
Glu	4.3	4
Gly	5.1	5
Ala	2.0	2
Val	1.0	1
Leu	1.0	1
Lys	1.3	1
His	2.2	2

Co-injection of synthetic FPA (Aα 1-16 peptide) and FPB (Bβ 1-14 peptide) with the TCA-soluble peptides revealed the absence of peptides with similar retention times to FPA and FPB in the peptide mixture (Fig 18A). The presence of FPA- and FPB-containing fragments was determined by adding thrombin to the peptide mixture. Treatment of the TCA soluble peptides with thrombin resulted in the cleavage of three peptides with retention times of 37.6, 38.3 and 38.8 min and the generation of a peptide with a retention time equivalent to that of FPA (Fig 18B). No generation of FPB was observed during this treatment with thrombin (Fig 18B). When the degradation mixture, prior to the precipitation of the large molecular mass degradation products with TCA, was treated

with thrombin, peptides with equivalent retention times to both FPA and FPB were generated (Fig 18C). The amount of FPA generated in this manner (as judged by peak heights and area under the peak) appeared to equal the amount of FPA generated when the TCA-soluble mixture was treated with thrombin (Fig 18B). When the peptides, from the TCA-soluble mixture, with retention times of 37.6, 38.3 and 38.8 min were individually isolated and treated with thrombin, only the peptide with the retention time of 38.3 min could generate FPA. This peptide was observed in the TCA-soluble peptide mixture after a 5 min incubation of fbg with the PMA-stimulated conditioned medium. The amino acid composition of this peptide, with a retention time of 38.3 min, was found to be identical to that of peptide Aα1-21, based on the known amino acid sequence data for amino acids at positions 1-21 from the N-terminus of the Aα chain (Table 13). Due to the present supposition that the release of the Aα1-21 peptide is a specific characteristic of the action of neutrophil granule elastase (Weitz *et al.*, 1986) it was necessary to conduct further experiments to confirm that the Aα1-21 peptide is also generated by a neutral membrane-associated neutrophil protease, distinct from granule elastase. Treatment of the TCA-soluble peptide mixture with HNE showed many of the peptides to be HNE sensitive, including those with the retention times of 37.6 and 38.8 min (Fig 18D). The peptide with the retention time of 38.3 min was, however, resistant to digestion by HNE (Fig 18D). This was further confirmed by the isolation of this peptide and subsequent treatment with HNE. When the elastase specific inhibitor, MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 M), known to block Aα1-21 formation by HNE (Weitz *et al.*, 1986), was included in the reaction with neutrophil conditioned medium, several peptides, including the one with the retention time of 38.3 min, were observed (Fig 18E). The amino acid composition of this peptide (eluted at 38.3 min) was confirmed to be that of Aα1-21 (Table 13) and the yield of the peptide, determined by peak heights and area under the peak, was more than 98% of that in the absence of the inhibitor. In contrast, MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 M) completely inhibited fbg digestion by HNE (Fig 18F). When undigested fbg was treated with HNE, many peptides were generated (Fig 18G) with retention times different to those generated by the neutrophil conditioned medium (Fig 18A). When these HNE generated peptides were treated with thrombin a peptide with a retention time of 38.3 min was cleaved and a peptide with a retention time equivalent to that of pure FPA was generated (Fig 18H). This HNE generated peptide, when isolated, co-eluted with the peptide with a retention time of 38.3 min produced by neutrophil conditioned medium degradation of fbg pre-(Fig 18A) and post-(Fig 18D) HNE treatment.

Results identical to those described above were achieved when the enzyme source used to degrade fbg was either neutrophil cytoskeleton or neutrophil membrane preparations (not shown).

Table 13. Amino acid analysis of the peptide with an elution time of 38.3 min. Amino acid sequence: Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val. This corresponds to residues 1-21 from the N-terminus of the Aα chain of fbg.

Amino acid	Residues found	Theoretical value
Asp	1.9	2
Ser	1.3	1
Glu	2.1	2
Pro	1.3	1
Gly	6.2	6
Ala	2.0	2
Val	3.0	3
Leu	1.0	1
Phe	1.0	1
Arg	2.1	2

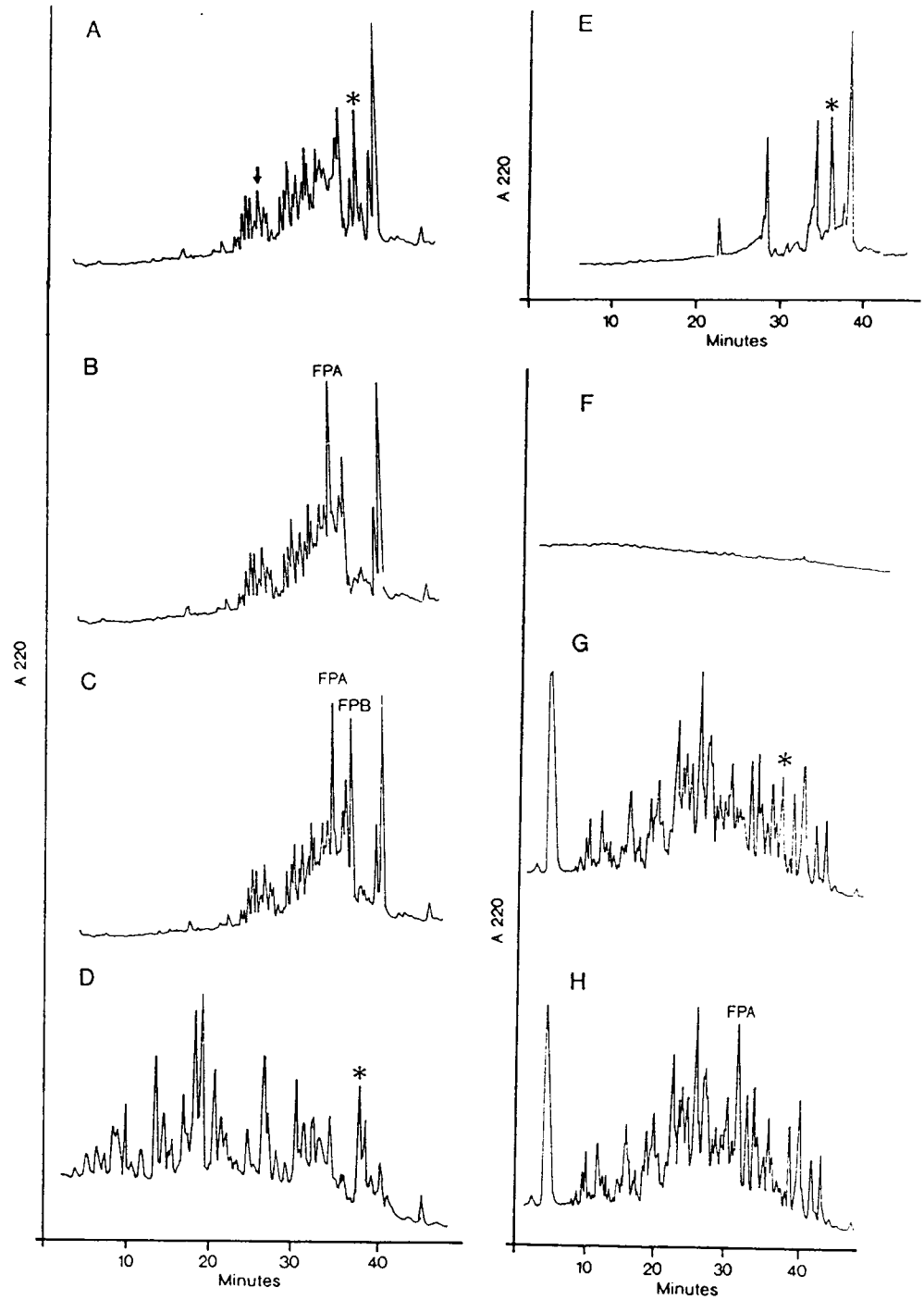

Figure 18. H.p.l.c. separation of the trichloractic acid-soluble fibrinogen peptides. Unlabelled fbg (2 mg) was incubated (37°C, 20 hr) with conditioned medium from $5x10^6$ PMA-stimulated neutrophils (**a-e**) or HNE (2 μg/ml) (**f-h**) and the TCA-soluble peptides obtained were separated using a C18 reverse phase column (flow rate 1 ml/min) (as described in the Materials and methods section). An asterisk indicates the peptide with an elution time of 38.3 min containing a thrombin sensitive site. (**a**) Peptides generated by a 20 hr digestion period. (↓) indicates the position at which a peptide with an amino acid composition corresponding to the amino acid sequence between positions 395-411 from the N-terminus of the γ chain was eluted. (**b**) Effect of treating the peptides shown in (**a**) with thrombin (2 i.u./ml, 1 hr, 37°C). FPA indicates the position at which the synthetic fibrinopeptide A would elute when co-injected with this peptide mixture. (**c**) Nature of the peptides generated by treating the 20 hr degradation mixture with thrombin (2 i.u./ml, 1 hr, 37°C) before stopping the reaction with TCA. FPA and FPB indicate the positions at which the synthetic fibrinopeptide A and B would be eluted when co-injected with the peptide mixture. (**d**) Treatment of the peptides shown in (**a**) with HNE (2 μg/ml, 2 hr, 37°C). (**e**) Peptides generated [as described in (**a**)] in the presence of MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 M). (**f**) Profile when the reaction contained HNE (2 μg/ml) and MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 M). (**g**) Nature of the peptides generated by HNE during a 2 hr digestion period. (**h**) Treatment of the peptides in (**g**) with thrombin (2 i.u./ml, 1 hr, 37°C). FPA indicates the position at which a synthetic fibrinopeptide A would be eluted when co-injected with this peptide mixture.

5.A.3. DISCUSSION

This study demonstrates the ability of a neutral, serine protease, associated with isolated neutrophil membranes, cytoskeletons and the conditioned medium from PMA-stimulated neutrophils, to cleave fbg, time and concentration dependently, to a non-clottable form. The fbg-degrading protease within each of these three neutrophil fractions was found to migrate on SDS-PAGE as four discrete bands of molecular mass values 209 kDa, 316 kDa, 398 kDa and 501 kDa. It has been proposed from our studies characterising this protease (see Chapter 4) that it may be located at sites of interaction of the membrane and the cytoskeleton.

The high molecular mass fbg products generated by the action of this neutral protease from the three neutrophil fractions were found to be distinctly different, on the basis of molecular mass, to the fbg products generated by plasmin, neutrophil lysosomal enzymes and pure HNE. In addition, the terminal fbg products generated by the PMA-stimulated neutrophil conditioned medium were sensitive to plasmin digestion. In contrast to plasmin degradation, the generation of the terminal fbg products by the neutrophil conditioned medium was insensitive to the presence or absence of Ca^{2+}. Fibrinogenolysis by the three neutrophil fractions appeared to be a result of elastase-like proteolytic activity as this degradation was partially inhibited by the elastase inhibitors, MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl and α_1AT. These inhibitors completely inhibited elastase degradation of fbg (Weitz *et al.*, 1986; Wright *et al.*, 1988; Weitz *et al.*, 1987; Weitz *et al.*, 1992). No inhibition of the fibrinogenolytic activity of the neutrophil membranes, cytoskeleton and conditioned medium could be achieved with other elastase inhibitors or inhibitors of plasmin and cathepsin G.

In the presence or absence of $CaCl_2$, simultaneous early cleavage of all three constituent chains of fbg, by the proteolytic activity associated with neutrophil membrane, cytoskeleton and conditioned medium fractions, gave rise to unclottable fbg products which do not exhibit anticoagulant activity. The initial prolongation of thrombin clotting time was exhibited by a fbg degradation product with an apparent molecular mass of 330 kDa which retains a partial capacity to form crosslinked fibrin. The prolongation of clotting time of this 330 kDa fbg product appears to result from the degradation of the Aα chain to a molecular mass of 45 kDa, and partial cleavage of the Bβ and γ chains to molecular mass values of 48 kDa and 38 kDa, respectively. The inability to form γ-γ dimers and loss of coagulability, that is characteristic of the fbg degradation product with an apparent molecular mass of 290 kDa, is the result of extensive cleavage of the Aα, Bβ and γ chains to apparent molecular mass values of 42 kDa, 48 kDa and 32 kDa respectively. This is in contrast

to fbg degradation by plasmin where the Aα chain is degraded rapidly followed by the Bβ chain and the γ chain is relatively resistant to degradation, especially in the presence of $CaCl_2$. Also, the plasmin generated fbg terminal products exhibit prolonged clotting times but are still able to form γ-γ dimers (Pizzo *et al.*, 1972).

As mentioned above, the fbg products generated by the neutrophil membrane-associated protease do not possess anticoagulant activity. Release of an Aα1-21 peptide within 5 min of digestion suggests that degradation of the Aα chain occurs from the N-terminus. On the other hand, the observation that FPB can be released from the high molecular mass split products at the end point of digestion and the detection of a 17 amino acid C-terminus γ chain peptide indicates fibrinogenolysis to occur from the C-terminal regions of the Bβ and γ chains. Such proteolysis of the Aα chain could account for the products lacking anticoagulant activity as the Aα1-21 peptide does not act as a competitive inhibitor of the action of thrombin on intact fbg (Larrieu *et al.*, 1972) and the C-terminal Gly-Pro-Arg-Val-Val sequence of the peptide has been shown to bind poorly to the primary polymerisation site on intact fbg (Laudano and Doolittle, 1980). The release of the 17 amino acid γ chain peptide was achieved by the cleavage of the bond between amino acids Ile and Gly at positions 394 and 395 from the N-terminus of the γ chain by the neutrophil membrane-associated protease. Although no attempt was made to detect other γ chain peptides released, particularly a peptide containing the primary fibrin polymerisation site at γ-363 (Shimizu *et al.*, 1992; Yamazumi and Doolittle, 1992), such a peptide may have been proteolytically removed. This is tenable since the neutrophil membrane protease has been shown to cleave bonds between Ala and Ser (Shephard *et al.*, 1990) which are found at positions 357 and 358 from the N-terminus of the γ chain. It is possible, however, that removal of the 17 amino acid peptide may be sufficient for loss of the appropriate structural configuration required for binding of the γ chain to complementary sites on the E domain of the fibrin monomer (Olexa *et al.*, 1981), which would account for the lack of anticoagulant activity of the degradation products. It has been shown that γ chain peptides 374-411 and 374-396 do not act as anticoagulants (Varadi and Scheraga, 1986).

In contrast, plasmin mediated fibrinogenolysis occurs from the C-terminus of the Aα chain, the N-terminus of the Bβ chain, with the γ chain being relatively resistant to degradation, and generates products with anticoagulant activity (Pizzo *et al.*, 1972; Doolittle, 1984; Ferguson *et al.*, 1975; Yamazumi and Doolittle, 1992; Varadi and Scheraga, 1986). Degradation of fbg by the neutrophil membrane-associated protease is also in contrast with that of neutrophil lysates and purified HNE

which cleave all three fbg chains from the N-terminus. Fbg products generated by the neutrophil lysate were also able to inhibit fibrin polymerisation (Bilezikian and Nossel, 1977; Gramse *et al.*, 1977; Plow and Edgington, 1975).

Several studies have concluded that HNE is solely responsible for the formation of an Aα1-21 peptide found in the serum of normal individuals and patients with α_1AT deficiency and this peptide has since been used as an assay for HNE activity in these α_1AT deficient patients (Weitz *et al.*, 1986; Weitz *et al.*, 1987; Weitz *et al.*, 1992). This study confirms the ability of pure HNE to generate this thrombin sensitive FPA-containing peptide and also demonstrates that an identical peptide can be generated by the proteolytic activity associated with the neutrophil membrane. The formation of this peptide by the neutrophil membrane-associated protease was insensitive to the inhibitor MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl that has been shown in several studies, and now confirmed here, to completely inhibit HNE formation of the Aα1-21 peptide (Weitz *et al.*, 1986; Wright *et al.*, 1988; Weitz *et al.*, 1987; Weitz *et al.*, 1992). The study of Weitz *et al.* (1987) also showed the generation of the Aα1-21 peptide, via an HNE-insensitive pathway, when non-stimulated neutrophils were incubated with fbg coated filters. This may be due to the action of the neutrophil membrane protease as it exhibits activity in non-stimulated neutrophils (Shephard *et al.*, 1989). It is, therefore, tenable that the existence of the Aα1-21 peptide in plasma may not be a specific marker of neutrophil granule associated elastase as previously believed (Weitz *et al.*, 1986; Wright *et al.*, 1988; Weitz *et al.*, 1987).

Only the bond between amino acids Val-21 and Glu-22 from the N-terminus of the Aα chain seems to be sensitive to both HNE and the proteolytic activity of the neutrophil membrane. Other elastase specific sites in fbg appear to be markedly different to those susceptible to cleavage by the neutrophil membrane-associated protease. This is concluded from the finding that the size of the high molecular mass degradation products and the h.p.l.c. profiles of the low molecular mass peptides, generated by each of these two proteases, are different.

5.B. ASSOCIATION OF FIBRINOGEN WITH NEUTROPHILS AND THE MECHANISM OF FIBRINOGEN DEGRADATION DURING ASSOCIATION

5.B.1. INTRODUCTION

The interaction of neutrophils with fbg, which is associated with the process of fibrin deposition, is regulated by membrane cytoadhesive receptors of the β_2 and β_3 integrin families (Wright *et al.*, 1988; Loike *et al.*, 1991; Diamond and Springer, 1993; Gresham *et al.*, 1992; Zhou and Brown, 1993). The integrin adhesion molecules are composed of non-covalently associated subunit pairs, designated α and β, that mediate cell-matrix and cell-cell adhesion processes (Smyth *et al.*, 1993). The integrins are classified according to the β subunit which may associate with one or more α subunits. The leukocyte specific or β_2 integrins have a common β subunit (CD18) which may associate with one of three α subunits, CD11a, CD11b and CD11c, to form the heterodimers CD11a/CD18 (LFA-1), CD11b/CD18 (CR3, Mac-1) and CD11c/CD18 (p150,95) (Fig 4) (Smyth *et al.*, 1993). Recently neutrophils have been shown to possess a novel integrin receptor described as the leukocyte response integrin (LRI) which is most immunologically related to the β_3 integrin family (Gresham *et al.*, 1992; Zhou and Brown, 1993). Depending on the stimulus, different integrin receptors on the neutrophil may interact with different specific fbg recognition sequences contained within both soluble and immobilised fbg. Various stimuli which have been investigated include TNF-α and FMLP (Wright *et al.*, 1988; Loike *et al.*, 1991; Diamond and Springer, 1993; Zhou and Brown, 1993). Fbg recognition sequences which bind to these integrin receptors have thus far been identified to include R-G-D, G-P-R and K-Q-A-G-D-V (Wright *et al.*, 1988; Loike *et al.*, 1991; Gresham *et al.*, 1992; Altieri *et al.*, 1988; Altieri *et al.*, 1993).

This study attempts to identify the neutrophil receptor which interacts with fbg leading to its degradation by a neutrophil membrane-associated protease. The association of fbg with the neutrophil surface, with concomitant degradation, will be characterised and the nature of the fbg associated with the neutrophil and the consequence of such association on subsequent neutrophil interactions with immobilised fbg will also be examined.

5.B.2. RESULTS

5.B.2.1. Degradation of [^{125}I]-labelled fibrinogen by neutrophils

Degradation of [^{125}I]-labelled fbg was observed upon incubation with both PMA-stimulated and non-stimulated neutrophils ($5x10^6$) and the conditioned medium from PMA-stimulated and non-stimulated neutrophils ($5x10^6$) (Fig 19).

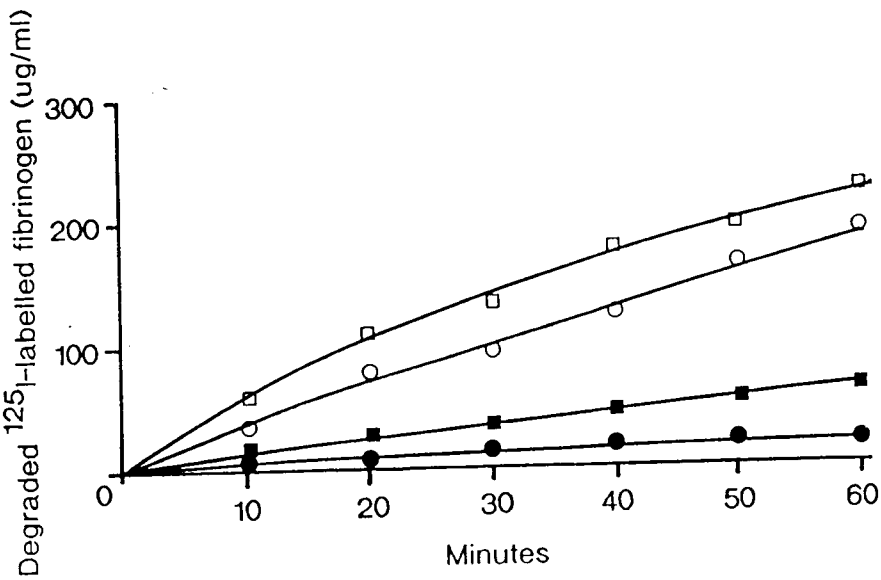

Figure 19. Degradation of [^{125}I]-labelled fibrinogen by neutrophils and neutrophil conditioned medium. Reactions (1 ml) contained [^{125}I]-labelled fbg (1 mg) and either PMA-stimulated (□) or non-stimulated neutrophils ($5x10^6$) (○) or the conditioned medium from PMA-stimulated (■) or non-stimulated neutrophils ($5x10^6$) (●). Degradation was measured as 10% (w/v) TCA-soluble peptides at the indicated time points and was expressed as μg [^{125}I]-labelled fbg/ml. Ten experiments gave results within 10% of these.

The generation of TCA-soluble [^{125}I]-labelled fbg peptides by PMA-stimulated neutrophils at 60 min was, however, 3.6 times greater than that by the PMA-stimulated neutrophil conditioned medium while non-stimulated neutrophils generated approximately 9 times more TCA-soluble products than the conditioned medium from these cells at 60 min (Fig 19). Neutrophil-mediated degradation of fbg was increased 1.3 times by stimulating the cells with PMA and the generation of TCA-soluble [^{125}I]-labelled fbg peptides by the PMA-stimulated conditioned medium at 60 min was 3 times greater than that of the conditioned medium generated from non-stimulated cells (Fig 19). Incubation of [^{125}I]-labelled fbg with PMA-stimulated neutrophils or the conditioned medium from these cells yielded TCA-soluble [^{125}I]-labelled fbg degradation products within 2 min (Fig 19). Results within 1% of those shown in Fig 19 were found with 10 different batches of neutrophils. The neutrophil-mediated degradation occurred faster in the presence of Ca^{2+} whereas the conditioned medium degradation was not affected by the chelation of Ca^{2+} (Table 14). SDS-PAGE (5-20% non-reduced and 5-20% reduced) analysis revealed that the apparent molecular mass of the [^{125}I]-labelled fbg products generated by PMA-stimulated and non-stimulated neutrophils were identical to those generated by the neutrophil conditioned medium from PMA-stimulated and non-stimulated cells (Fig 20A and B) The time course of [^{125}I]-labelled fbg degradation depicted in Fig 20B confirms the increased degradation rate of [^{125}I]-labelled fbg in the presence of the cells when compared to that in the presence of the neutrophil conditioned medium.

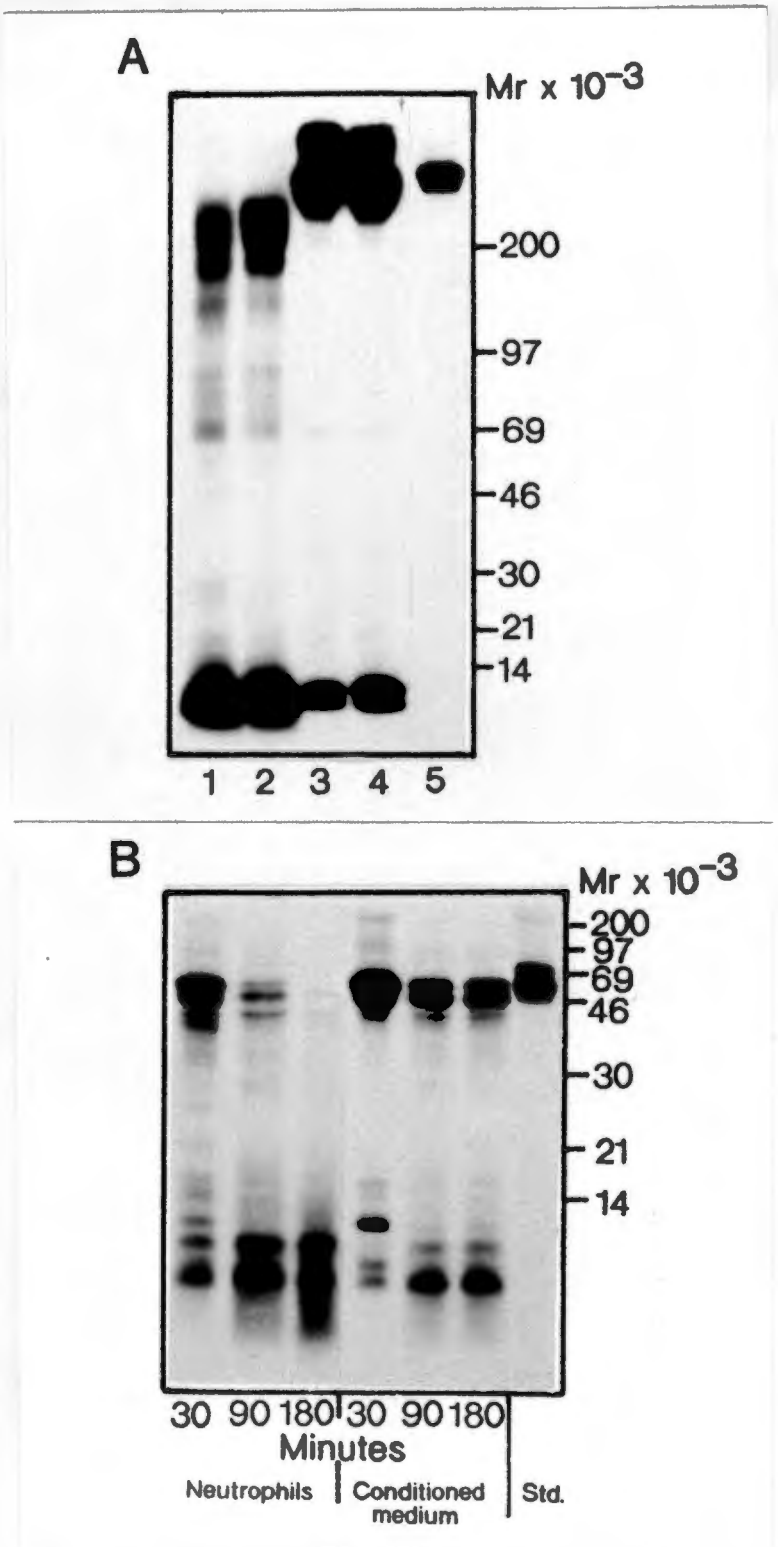

Figure 20. The nature of [^{125}I]-labelled fibrinogen degradation products generated by neutrophils and neutrophil conditioned medium in the presence and absence of PMA [^{125}I]-labelled fbg (1 mg) was incubated (37°C) with either PMA-stimulated or non-stimulated neutrophils ($5x10^6$) or the conditioned medium from either PMA-stimulated or non-stimulated neutrophils ($5x10^6$). The [^{125}I]-labelled fbg products thus generated were analysed by SDS-PAGE followed by autoradiography, as described in the Materials and methods. (**A**) SDS-PAGE (5-20% non-reduced) indicating [^{125}I]-labelled fbg degradation at 30 min. Track 1: non-stimulated neutrophil-mediated degradation, track 2: PMA-stimulated neutrophil-mediated degradation, track 3: non-stimulated conditioned medium degradation and track 4: PMA-stimulated conditioned medium degradation. Track 5: Undigested [^{125}I]-labelled fbg. (**B**) SDS-PAGE (5-20% reduced) indicating PMA-stimulated neutrophil-mediated degradation and PMA-stimulated neutrophil conditioned medium degradation of [^{125}I]-labelled fbg occurring over a period of 30 min to 3 hr. Undigested [^{125}I]-labelled fbg is indicated in the track marked Std.

Table 14. The influence of Ca^{2+} on the degradation of [^{125}I]-labelled fibrinogen. PMA-stimulated and non-stimulated neutrophils ($5x10^6$) and the conditioned medium from $5x10^6$ PMA-stimulated and non-stimulated neutrophils were incubated (37°C) with [^{125}I]-labelled fbg (1 mg) in the absence or presence of EGTA (10 mM). Degradation was measured at 30 min and 3 hr as 10% (w/v) TCA [^{125}I]-labelled fbg peptide formation and was expressed as μg/ml.

	Degraded [^{126}I]-labelled fibrinogen (μg/ml)			
	NEUTROPHILS			
	+PMA		- PMA	
Time (min)	+ Ca^{2+}	- Ca^{2+}	+ Ca^{2+}	- Ca^{2+}
30	83	29	54	18
180	420	280	300	53
	CONDITIONED MEDIUM			
30	36	35	13	18
180	180	170	25	26

Degradation of [^{125}I]-labelled fbg by PMA-stimulated neutrophils was inhibited by 86%, 50% and 98% by α_1AT (1.5 mg/ml), MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (1mM) and AEBSF (1mM), respectively, within a 30 min period (Table 15). No inhibition of [^{125}I]-labelled fbg by PMA-stimulated neutrophils was noted with leupeptin (20 μM) (Table 15). A similar inhibition of degradation by non-stimulated neutrophils was obtained with these inhibitors (not shown).

Table 15. Influence of inhibitors on the degradation of [^{125}I]-labelled fibrinogen by PMA-stimulated neutrophils. PMA-stimulated neutrophils ($5x10^6$) were preincubated (37°C, 15 min) with various enzyme inhibitors whereafter the neutrophils were incubated (37°C, 30 min) with [^{125}I]-labelled fbg (1 mg). Degradation was measured as 10% (w/v) TCA [^{125}I]-labelled fbg peptide formation and was expressed in µg/ml.

Degraded [^{126}I]-labelled fibrinogen (µg/ml)		
Inhibitor	Concentration	Time 30 min
None		138
MeOSucAla$_2$ProValCH$_2$Cl	1mM	69
α_1-antitrypsin	1.5 mg/ml	19
Leupeptin	20 µM	138
AEBSF	1 mM	2

The rate of PMA-stimulated neutrophil-mediated degradation of [^{125}I]-labelled fbg was reduced, at 30 min, by 75% by the kinase inhibitors TFP (20 µM) and H7 (50 µM) (Table 16). The generation of [^{125}I]-labelled fbg degradation products by PMA-stimulated neutrophils was inhibited by 65% by 1-(5-chloronaphthalene-1 sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9, 20 µM) (Table 16). No inhibition of PMA-stimulated neutrophil-mediated degradation of [^{125}I]-labelled fbg was observed with the protein kinase C inhibitor, bisindolylmaleimide (Table 16). These inhibitors did not affect the viability of the cells.

The percentage inhibition of [^{125}I]-labelled fbg degradation by non-stimulated neutrophils was identical to that observed with the PMA-stimulated cells, for all the kinase inhibitors tested (Table 16).

Table 16. Influence of kinase inhibitors on neutrophil-mediated degradation of [^{125}I]-labelled fibrinogen. Reactions (1 ml) contained [^{125}I]-labelled fbg (1 mg) and either PMA-stimulated or non-stimulated neutrophils ($5x10^6$) that had been preincubated (37°C) for 15 min with or without the indicated inhibitor. Degraded fbg, measured at the indicated times as 10% (w/v) TCA-soluble peptides, was expressed as µg degraded [^{125}I]-labelled fbg/ml. Results are from a representative experiment.

Degraded fibrinogen (µg/ml)						
	+PMA			- PMA		
Time (min)	2	10	30	2	10	30
Inhibitor						
None	20	60	138	16	34	95
ML-9 (20 µM)	15	20	48	8	11	32
TFP (20 µM)	5	12	35	4	7	24
H7 (50 µM)	6	13	34	5	5	23
Bisindolylmaleimide (2 µM)	20	60	138	16	34	95

5.B.2.2. Influence of [^{125}I]-labelled fibrinogen on proteolytic activity in the extracellular medium during cellular degradation.

Degradation of [^{125}I]-labelled fbg by both PMA-stimulated and non-stimulated neutrophils is notably faster than that of the conditioned medium prepared from these cells (Fig 19). A release of proteolytic activity into the extracellular medium during the incubation of neutrophils with fbg could account for this observation. To investigate this possibility, the rate at which [^{125}I]-labelled fbg continues to degrade, following the removal of the cells, was compared with that by PMA-stimulated cells and PMA-stimulated neutrophil conditioned medium. When the incubation period of PMA-stimulated neutrophils with [^{125}I]-labelled fbg was less than 30 min, degradation of [^{125}I]-labelled fbg in the extracellular medium was slower than that in the presence of cells, but faster than that by PMA-stimulated neutrophil conditioned medium (Table 17). However, if the incubation period of cells with [^{125}I]-labelled fbg was longer than 30 min, prior to removal of the cells, the rate of extracellular degradation was equal to that in the presence of cells (Table 17).

Similarly, [^{125}I]-labelled fbg is able to mediate the release of fbg-degrading activity from non-stimulated neutrophils. As for the stimulated cells, a 30 min incubation period of [^{125}I]-labelled fbg with non-stimulated neutrophils resulted in the degradation of [^{125}I]-labelled fbg in the cell free medium being equal to that in the presence of non-stimulated neutrophils and greater than that of the conditioned medium from non-simulated neutrophils (Table 17).

Table 17. Proteolysis of [^{125}I]-labelled fibrinogen in the extracellular medium during neutrophil association with fibrinogen. Reactions (1 ml) contained [^{125}I]-labelled fbg (1 mg) and either the conditioned medium from $5x10^6$ neutrophils (PMA-stimulated and non-stimulated) or $5x10^6$ neutrophils (PMA-stimulated or non-stimulated). Degradation was measured as 10% (w/v) TCA-soluble peptides and expressed as µg [^{125}I]-labelled fbg/ml. Neutrophil-mediated or conditioned medium degradation was measured at the indicated times (••). Degradation in the extracellular medium, obtained by removing the cells from the neutrophil-mediated reaction at various times (••), was measured at the indicated times (*).

Degraded fibrinogen (µg/ml)							
+ PMA							
	Conditioned Medium	Neutrophil Mediated	Extracellular Medium				
Time			20*	30	40	60	90
2••	6	12	60	70	90	120	130
10	18	60	80	100	120	140	170
20	25	110	-	120	130	160	190
30	32	140	-	-	170	210	240
40	44	170	-	-	-	220	240
60	60	220	-	-	-	-	240
- PMA							
2	5	10	40	45	60	80	110
10	8	35	50	80	90	130	150
20	11	77	-	90	90	130	160
30	14	90	-	-	120	140	190
40	18	120	-	-	-	180	210
60	20	180	-	-	-	-	210

5.B.2.3. Influence of AEBSF on the association of [^{125}I]-labelled fibrinogen with neutrophils

The association of [^{125}I]-labelled fbg with non-stimulated and PMA-stimulated neutrophils was investigated in the presence and absence of the inhibitor AEBSF. The serine protease inhibitor, AEBSF (1 mM), has been demonstrated to inhibit neutrophil-mediated degradation of [^{125}I]-labelled fbg by 98% (see above).

In the absence of AEBSF, the association of [^{125}I]-labelled fbg with neutrophils ($5x10^6$) was fast, occurring as early as 2 min (Fig 21). The association of [^{125}I]-labelled fbg with PMA-stimulated neutrophils was far greater than the association with non-stimulated neutrophils and reached a steady state within 30 min (Fig 21) via a saturable process, as shown by concentration dependence curves (Fig 22A). Over the concentration range of [^{125}I]-labelled fbg offered to the neutrophils, the non-specific association of [^{125}I]-labelled fbg with the neutrophils, in the presence of a 100 fold molar excess of unlabelled fbg, varied from 2-11% of the total [^{125}I]-labelled fbg associated with the cells. Specific association was calculated by subtracting the non-specific association from the total [^{125}I]-labelled fbg associated with the cell. When 1 mg of [^{125}I]-labelled fbg was offered to $5x10^6$ non-stimulated or PMA-stimulated cells in 1 ml and in the presence of Ca^{2+}, the specific association reached a steady state. At saturation 600 ng [^{125}I]-labelled fbg was associated with $5x10^6$ PMA-stimulated neutrophils with half-maximal saturation occurring at 200 µg [^{125}I]-labelled fbg/ml (Fig 22A). This association of [^{125}I]-labelled fbg with PMA-stimulated neutrophils, was not influenced in the absence of Ca^{2+}. By contrast, at 30 min only 290 ng [^{125}I]-labelled fbg was associated with $5x10^6$ non-stimulated neutrophils at saturation and in the presence of Ca^{2+}, with half-maximal saturation occurring at 260 µg [^{125}I]-labelled fbg/ml (Fig 22A). These results varied by less than 10% for 10 different experiments.

In the presence of the serine protease inhibitor AEBSF (1mM) less [^{125}I]-labelled fbg associated specifically and saturably with PMA-stimulated neutrophils than in the absence of AEBSF (Fig 22A). Similar to the situation in the absence of AEBSF, a steady state was reached in 30 min, but in the presence of AEBSF (1 mM) only 400 µg [^{125}I]-labelled fbg/ml was required to reach this saturation. At saturation 80 ng [^{125}I]-labelled fbg was associated with $5x10^6$ PMA-stimulated neutrophils with half-maximal saturation occurring at 78 µg [^{125}I]-labelled fbg/ml (Fig 22A). By contrast, association of [^{125}I]-labelled fbg with the non-stimulated neutrophils, in the presence of AEBSF (1 mM), reached saturation at 1 mg [^{125}I]-labelled fbg, but at saturation the amount of fbg bound was only marginally less than that with the PMA-stimulated neutrophils (Fig 22A). 75 ng [^{125}I]-labelled fbg was associated with $5x10^6$ non-stimulated neutrophils at saturation with half-maximal saturation occurring at 330 µg [^{125}I]-labelled fbg/ml (Fig 22A). Four different experiments gave results within 10% of these.

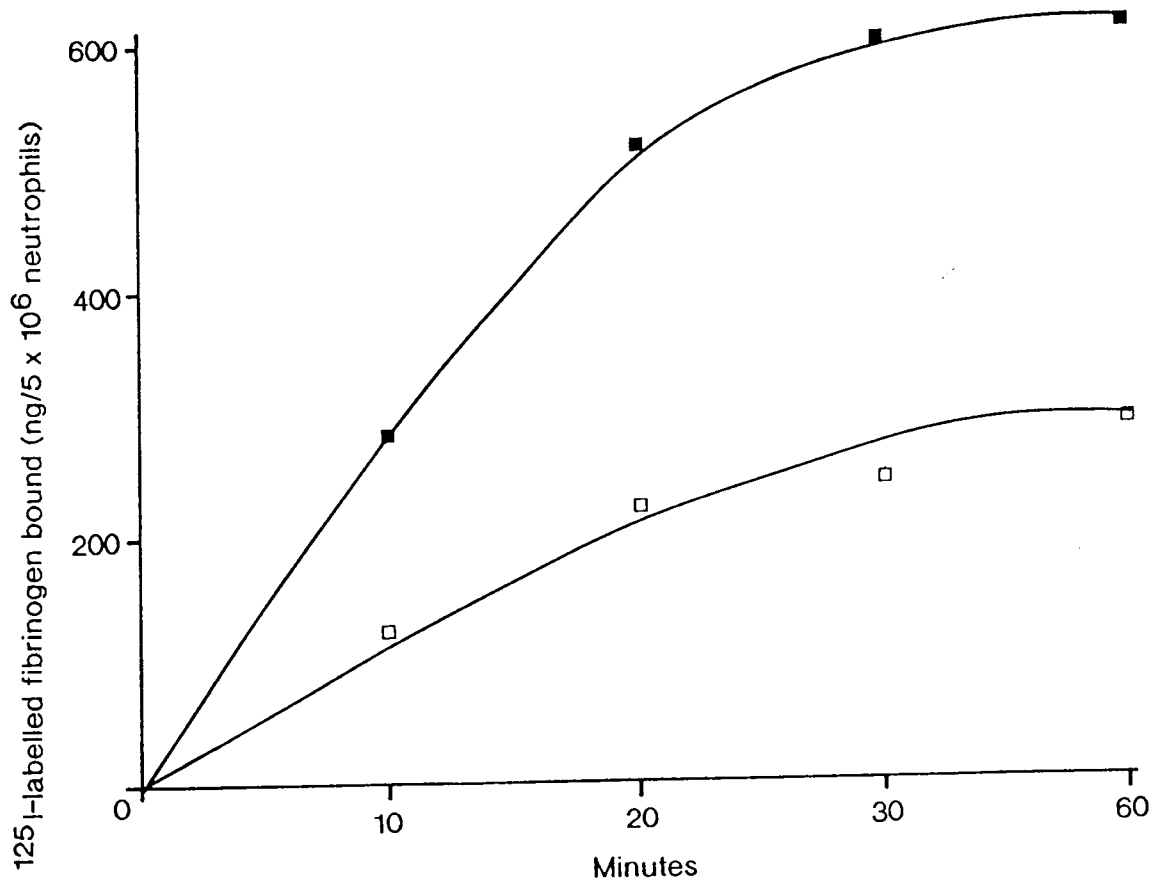

Figure 21. Specific association of [^{125}I]-labelled fibrinogen with neutrophils occurring over time. [^{125}I]-labelled fbg (1 mg) was incubated (37°C) with either PMA-stimulated (■) or non-stimulated (□) neutrophils ($5x10^6$). At the indicated times over a period from 2 to 60 min, the reactions were stopped and the specific association of [^{125}I]-labelled fbg was calculated as described in the Materials and methods. Each value is the mean of duplicates which did not differ by more than 10%. Ten experiments gave results within 10% of these.

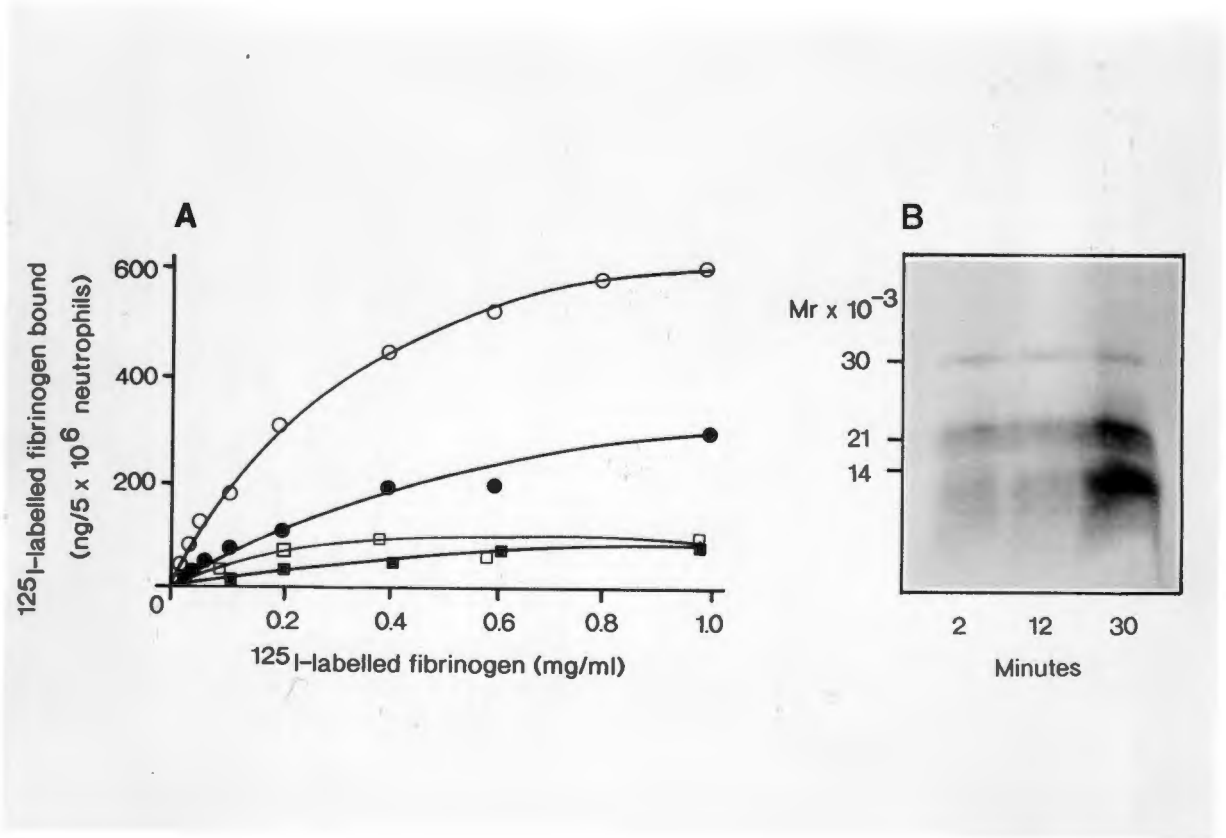

Figure 22. Specific association of [^{125}I]-labelled fibrinogen with PMA-stimulated neutrophils as a function of concentration. A. [^{125}I]-labelled fbg was incubated (37^{o}C, 30 min) with either PMA-stimulated neutrophils (5x10^{6}) in the absence (○) or presence (□) of the inhibitor AEBSF (1 mM) or non-stimulated neutrophils (5x10^{6}) in the absence (●) or presence (■) of the inhibitor AEBSF (1 mM). Specific association of [^{125}I]-labelled fbg was calculated as described in Methods. Each value is a mean of duplicates which did not differ by more than 10%. **B.** PMA-stimulated neutrophils (5x10^{6}) were incubated (37^{o}C) with [^{125}I]-labelled fbg (1 mg) in a final volume of 1 ml and the cell associated [^{125}I]-labelled fbg was analysed by SDS-PAGE (5-20% non-reduced) at 2, 12 and 30 min.

5.B.2.4. Nature of the [^{125}I]-labelled fibrinogen degradation products associated with the neutrophil

The nature of the [^{125}I]-labelled fbg degradation products associated with PMA-stimulated neutrophils, in the absence of AEBSF, was ascertained by SDS-PAGE (5-20%, non-reduced) followed by autoradiography. No intact fbg was found to be associated with the cell. The [^{125}I]-labelled fbg degradation products observed to be associated with the neutrophil appeared to have apparent molecular mass values of 30 kDa or less (Fig 22B). Products with identical molecular mass values were found to associate with the non-stimulated neutrophils (not shown).

5.B.2.5. Influence of fibrinogen degradation products on the association of [^{125}I]-labelled fibrinogen with PMA-stimulated neutrophils

Since the size of the degradation products associated with non-stimulated and PMA-stimulated neutrophils were identical, the specificity of the interaction of the neutrophil derived [^{125}I]-labelled fbg degradation products, with apparent molecular mass values of 30 kDa and less, with PMA-stimulated neutrophils was investigated. To accomplish this, the ability of TCA-soluble fbg products to competitively inhibit the association of [^{125}I]-labelled fbg with PMA-stimulated neutrophils, in the absence of AEBSF, was assessed. It was found that, when used at a concentration of 25 μg/ml, the unlabelled fbg degradation products inhibited the association of [^{125}I]-labelled fbg (600 ng/5x10^6 cells) with PMA-stimulated neutrophils by 50% (Fig 23). The fbg degradation products were, however, not shown to have any effect on the degradation of the intact [^{125}I]-labelled fbg.

In an attempt to identify specific fbg sequences capable of associating with the neutrophil surface, various synthetic fbg peptides were included in the incubation of [^{125}I]-labelled fbg with PMA-stimulated neutrophils, in the absence of AEBSF. No modulation of the association of [^{125}I]-labelled fbg degradation products with neutrophils was observed when the synthetic peptides (all at 1 mg/ml) G-P-R-P, R-G-D-S, and H10, H12 or H15 (corresponding to residues 399-411, 400-411 and 398-411 of the γ chain respectively, all including the K-Q-A-G-D-V sequence), FPA (Aα 1-16) and FPB (Bβ 1 -14) were present in the reaction.

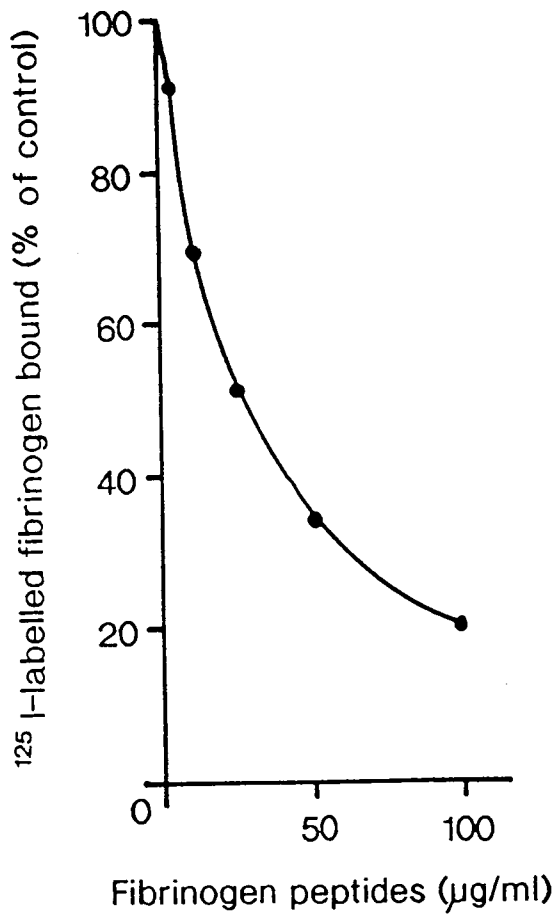

Figure 23. Influence of the neutrophil-derived fibrinogen degradation products on the association of [^{125}I]-labelled fibrinogen with PMA-stimulated neutrophils. PMA-stimulated neutrophils ($5x10^6$) were incubated (37°C, 30 min) with [^{125}I]-labelled fbg (1 mg) in the absence and presence of neutrophil-derived fbg products (0-100 µg) in a final volume of 1 ml. Neutrophil associated [^{125}I]-labelled fbg in the presence of fbg products is expressed as a percentage of neutrophil-associated [^{125}I]-labelled fbg in the absence of fbg degradation products (control, 600 ng/$5x10^6$ cells, 30 min). Values are the mean of duplicates which did not differ by more than 10%. This experiment is representative of three separate experiments.

5.B.2.6. Influence of monoclonal antibodies on PMA-stimulated neutrophil association and degradation of [^{125}I]-labelled fibrinogen

In order to elucidate the neutrophil receptor involved in the association of [^{125}I]-labelled fbg and neutrophil-derived [^{125}I]-labelled fbg degradation products with PMA-stimulated neutrophils, monoclonal antibodies directed against known neutrophil receptors were investigated. These experiments were conducted in the absence and presence of AEBSF (1 mM). The monoclonal antibodies used were directed against CD16 (the neutrophil low avidity Fc receptor), CD11a, CD11b, CD11c, CD18 and the intact gpIIb/IIIa complex (CD41a). The concentration of monoclonal antibodies required to saturate these receptors on PMA-stimulated neutrophils was determined by flow cytometry.

When saturating concentrations of the monoclonal antibodies against CD16, CD11a, CD11b, CD11c and CD18 were preincubated with PMA-stimulated neutrophils, in the absence of AEBSF, inhibition of the association of [^{125}I]-labelled fbg with the neutrophils was noted (Table 18). The monoclonal antibodies (obtained from Sanbio) directed against CD16, CD11a, CD11b, CD11c and CD18 inhibited the specific association of [^{125}I]-labelled fbg (600 ng/5x10^6 cells at 30 min, Fig 22) by 25%, 31%, 35%, 46% and 10%, respectively. The monoclonal antibodies directed against CD11a, CD11c and CD18 (manufactured by Serotec) inhibited the specific association of [^{125}I]-labelled fbg with PMA-stimulated neutrophils by 40%, 30% and 10%, respectively (Table 18). Monoclonal antibodies OKM1 and OKM10 (directed against CD11b) inhibited this specific association by 22% and 18%, respectively, and no inhibition was achieved with saturating concentrations of monoclonal antibodies directed against CD41a (Sanbio) (Table 18). Two different monoclonal antibodies recognising two different epitopes of CD41a gave the same result. A combination of OKM1, OKM10 and antibodies to CD18, CD11a, CD11b and CD11c inhibited the association of [^{125}I]-labelled fbg with the neutrophils by 60% (Table 18).

No modulation of the association of [^{125}I]-labelled fbg with either non-stimulated or PMA-stimulated neutrophils in the presence of AEBSF (1 mM) (80 ng/5x10^6 neutrophils in 30 min, Fig 22) could be achieved with any of the monoclonal antibodies tested.

Only the monoclonal antibody directed against CD11c (from Sanbio and Serotec) was shown to have any modulatory effect on the degradation of [^{125}I]-labelled fbg by the PMA-stimulated neutrophils (Table 18). In the absence of the anti-CD11c and AEBSF, 140 µg TCA-soluble [^{125}I]-labelled fbg peptides were generated from 1 mg [^{125}I]-labelled fbg by 5x10^6 PMA-stimulated

neutrophils, in 30 min. In the presence of the anti-CD11c (and the absence of AEBSF), 30 μg TCA-soluble [^{125}I]-labelled fbg peptides were generated by 5x10^6 PMA-stimulated neutrophils, in 30 min (Table 18). Thus, in the presence of the monoclonal antibody directed against CD11c the PMA-stimulated neutrophil-mediated degradation of [^{125}I]-labelled fbg is reduced to a rate similar to that of neutrophil conditioned medium (Fig 19). When the antibody to CD11c and AEBSF (1mM) were both present in the reaction, degradation of [^{125}I]-labelled fbg by PMA-stimulated neutrophils was inhibited by 100%. None of the other monoclonal antibodies tested altered the rate of neutrophil-mediated [^{125}I]-labelled fbg degradation (Table 18).

Table 18. Influence of monoclonal antibodies, directed against various neutrophil receptors, on PMA-stimulated neutrophil association and degradation of [^{125}I]-labelled fibrinogen. [^{125}I]-labelled fbg (1 mg) was incubated (37°C, 15 min) with PMA-stimulated neutrophils (5x10^6) and monoclonal antibodies (from Sanbio or Serotec) to various neutrophil receptors, in the presence and absence of the inhibitor AEBSF (1 mM). In one instance (*) a combination of antibodies directed against CD11a, CD11b, CD11c and CD18 were used (as described in the Materials and methods). The specific association of [^{125}I]-labelled fbg with the neutrophils was expressed in ng/5x10^6 neutrophils and the degradation was measured as 10% (w/v) TCA-soluble [^{125}I]-labelled fbg peptide formation and expressed as μg/ml.

	ng bound fibrinogen bound /5x10^6 cells			degraded fibrinogen (μg/ml)	
	+ AEBSF	- AEBSF		+ AEBSF	- AEBSF
Monoclonal Antibody	Serotec or Sanbio	Serotec	Sanbio	Serotec or Sanbio	Serotec or Sanbio
anti-CD11a	80	360	414	3	140
anti-CD11b	80	-	390	3	140
anti-CD11c	80	420	325	0	30
anti-CD18	80	540	540	3	140
OKM1	80	-	468	3	140
OKM10	80	-	492	3	140
All of above *	80	-	240	3	140
anti-CD41a	80	-	600	3	140
anti-CD16	80	-	450	3	140
None	80	600	600	3	140

5.B.2.7. The effect of the neutrophil-derived fibrinogen products on PMA-stimulated neutrophil adherence to immobilised fibrinogen

In the absence of AEBSF, and any neutrophil derived fbg products, 60% of the total number of neutrophils offered (2x10^6) adhered to the immobilised fbg. The neutrophil-derived fbg products, of molecular mass 30 kDa and less, concentration dependently inhibited the adherence of [^{51}Cr]-labelled neutrophils to immobilised fbg. Half maximal inhibition was achieved with 25 μg peptide/ml (Fig 24).

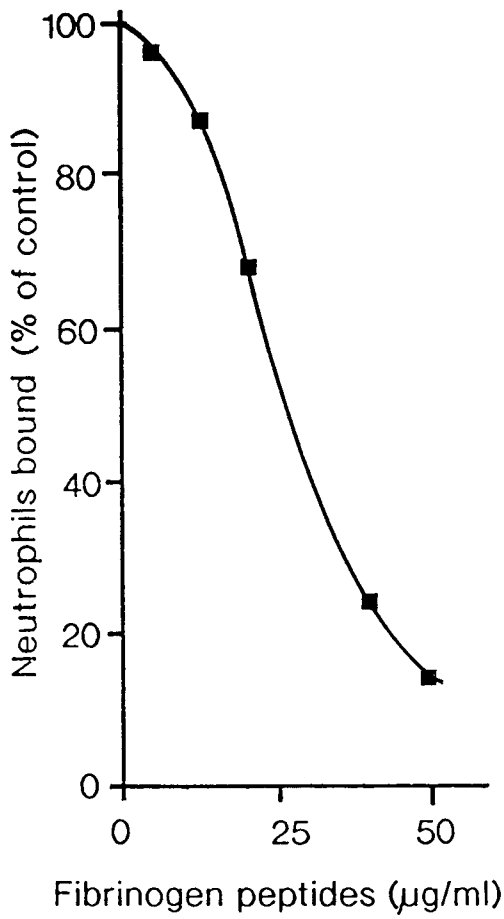

Figure 24. Influence of the neutrophil-derived fibrinogen degradation products on the adherence of [^{51}Cr]-labelled neutrophils to immobilised fibrinogen. [^{51}Cr]-labelled neutrophils ($2x10^6$) were preincubated (37°C, 30 min) with TCA-soluble fbg peptides at a concentration range of 0-50 µg/ml, as described in the Materials and methods. The neutrophils ($2x10^6$) were stimulated with PMA (5 ng/ml) and incubated (37°C, 30 min) with immobilised fbg. In the absence of the fbg degradation products $0.11x10^6$ PMA-stimulated neutrophils attached/fbg coated well and association, in the presence of the fbg products, was expressed as a percentage of this control figure. Values are the mean of quadruplicates which did not differ by more than 10%. This experiment is a representative from three separate experiments.

5.B.3. DISCUSSION

The interaction of neutrophils with immobilised and soluble fbg has been the topic of a number of investigations (Wright *et al.*, 1988; Loike *et al.*, 1991; Diamond and Springer, 1993; Gresham *et al.*, 1992; Zhou and Brown, 1993). A major feature of the interaction of neutrophils with immobilised fbg is the cleavage of the N-terminus of the Aα chain by elastase to generate the Aα1-21 peptide (Wright *et al.*, 1988). Studies describing the interaction of neutrophils with soluble fbg have not addressed concomitant degradation of this ligand.

This study demonstrates that fbg is degraded during an incubation of soluble [^{125}I]-labelled fbg with neutrophils. A neutrophil-mediated proteolytic process appears to contribute significantly to this process since fibrinogenolysis in the presence of cells was consistently faster than that by the neutrophil conditioned medium. As degradation of the ligand could be detected as early as 2 min upon incubation of fbg with neutrophils, the neutrophil-mediated fibrinogenolysis is likely to be due to a membrane-associated protease rather than internal cellular metabolism. Incubation of fbg with neutrophils caused considerable activation of the membrane-associated proteolytic activity *per se* with further activation being achieved by stimulating the cells with PMA. By contrast, CRP, which is another substrate for this membrane-associated protease (see Chapter 4, Shephard *et al.*, 1989), did not significantly upregulate this protease during association of CRP with non-stimulated neutrophils. Considerable activation was, however, observed during association of CRP with PMA-stimulated cells (see Chapter 4, Shephard *et al.*, 1989). Upregulation of the activity of a neutrophil surface-associated protease by both acute-phase and normal HDL has been demonstrated in a study investigating the interaction of HDL with neutrophils (Shephard *et al.*, 1987).

The apparent molecular mass of the fbg degradation products generated by neutrophils and neutrophil conditioned medium were similar. The fibrinogenolytic activity of the neutrophil conditioned medium has been shown (Chapter 5.A.) to be due to a high molecular mass membrane-associated protease which is released from the cell during the preparation of the conditioned medium (Chapter 4). Thus, degradation of fbg during its incubation with neutrophils is more than likely the result of the activation of this membrane-associated protease. The results in Chapter 4 suggested that the membrane-associated protease may be a submembrane protease localised at sites of attachment of the cytoskeleton with the cell membrane.

The upregulation of the activity of the neutrophil membrane-associated protease, that occurs following the interaction of fbg with neutrophils, appears to be the result of the activation of cellular kinases. It does not appear as if PKC is involved in this process as the specific PKC inhibitor, bisindolylmaleimide (Toullec *et al.*, 1991), did not inhibit neutrophil-mediated degradation of fbg. However, inhibition of fbg degradation by H7, TFP and ML-9, suggests that Ca^{2+}/calmodulin-dependent kinases, which are sensitive to these inhibitors, may be activated by fbg interaction with the neutrophil membrane. The inhibition of neutrophil-mediated fibrinogenolysis in Ca^{2+} depleted media may support the participation of Ca^{2+}/calmodulin-dependent kinases in the activation of the membrane-associated protease. However, the decreased neutrophil-mediated degradation of fbg in the absence of Ca^{2+} might reflect possible Ca^{2+} dependent binding of fbg to neutrophils. This does not, however, appear to be the case since neutrophil-mediated fibrinogenolysis is not reduced to that of conditioned medium in the absence of Ca^{2+} and association of fbg with PMA-stimulated neutrophils is not influenced in the absence of Ca^{2}, indicating Ca^{2+} independent binding of fbg to neutrophils. ML-9 inhibits actin-myosin interactions by the inhibition of phosphorylation of the myosin light chain by the myosin light chain kinase (MLCK) (Ito *et al.*, 1989). Thus, microfilament assembly may be important for the upregulation of the neutrophil membrane-associated proteolytic activity following the interaction of fbg with the neutrophil surface. An identical inhibition profile with these inhibitors, for both PMA-stimulated neutrophils and non-stimulated neutrophils, substantiates the findings that fbg association with the neutrophil *per se*, results in significant upregulation of the membrane associated protease. By contrast, the interaction of CRP with non-stimulated neutrophils did not activate cellular kinases. The observed activation of kinases during the incubation of CRP with PMA-stimulated neutrophils appears to be due to the action of PMA on the neutrophil (see Chapter 4, Shephard *et al.*, 1989).

The association of fbg with the neutrophil membrane of both non-stimulated and PMA-stimulated neutrophils occurred in a saturable and specific manner although stimulation with PMA appeared to increase the affinity of fbg binding. The association of fbg with neutrophils occurred at a position in close proximity to the CD11c, β_2 integrin receptor and resulted in, not only the upregulation of the membrane-associated protease, but also a time dependent release of this protease into the extracellular medium. The period required for maximal release of the protease may relate to the time required for the association of fbg with the neutrophil to reach equilibrium. Degradation of fbg by neutrophils, thus occurs both at the cell surface and in the extracellular medium.

Intact fbg could not be detected in association with the neutrophil as a result of neutrophil surface and subsequent extracellular-mediated degradation of fbg. The fbg products (of 30 kDa and less), however, associated specifically with the neutrophil Fc receptor, CD16 and the β_2 integrins, CD11a/CD18, CD11b/CD18 and CD11c/CD18. Although receptors recognised by monoclonal antibodies directed against the gpIIb/IIIa (β_3) receptor were expressed during PMA-stimulation (data not shown) no interaction of the degradation products with this receptor could be detected. The amino acid sequences within these fbg derived peptides do not appear homologous with K-Q-A-G-D-V or G-P-R, which are known to be ligand recognition sequences for CD11b/CD18 and CD11c/CD18, respectively (Wright *et al.*, 1988; Loike *et al.*, 1991), or R-G-D which is recognised as a common ligand recognition sequence for integrins (Smyth *et al.*, 1993). A 30 kDa plasmin-derived peptide (fragment D) has been shown to bind to the CD11b/CD18 receptor via a sequence within the γ chain (Gly-190 to Val-200) which does not contain any of the known ligand recognition sequences (Altieri *et al.*, 1988; Altieri *et al.*, 1993). The possibility exists that this sequence is present within the 30 kDa degradation product described in this study, but this was not explored.

The observed inhibition of neutrophil adherence to immobilised fbg by the neutrophil-derived fbg degradation products could modulate neutrophil interactions with extracellular matrix components as well as neutrophil β_2 integrin interactions with endothelial counter receptors ICAM-1 and ICAM-2. In addition, the interaction of the neutrophil-derived fbg degradation products with CD16 may modulate neutrophil Fc receptor function. When this is considered, together with the results which show that neutrophil fibrinogenolysis generates non-clottable fbg, it is tenable that neutrophil proteolysis of fbg could play an important role in the modulation of inflammatory processes through the regulation of fibrin deposition, neutrophil adhesion and migratory processes.

CHAPTER 6

SUMMARY AND CONCLUSION

The association of CRP with neutrophils and the concomitant degradation of this ligand by a PMA-upregulatable membrane-associated neutral protease has been shown in previous studies (Shephard *et al.*, 1989). The aim of this study has been to identify the cellular distribution of this protease and the mechanism of neutrophil-mediated degradation of CRP and another acute phase protein, fibrinogen, during association of these ligands with neutrophils.

A non-ionic detergent insoluble neutrophil fraction, containing proteins of the neutrophil cytoskeleton was found to contain this neutral protease. This may suggest that the enzyme is located within the cell as a submembrane protease, localised at sites of interaction of the cytoskeleton with the membrane. Size exclusion chromatography showed the protease to be of the approximate molecular mass 600 kDa and SDS-PAGE dissociated this protease into four distinct enzymatic species migrating to positions corresponding to the apparent molecular mass values of 209 kDa, 316 kDa, 398 kDa and 501 kDa.

Fibrinogen was also found to be a substrate for this protease. Degradation of fibrinogen by this neutrophil membrane-associated protease occurred in a manner distinct from other known fibrinogenolytic systems, including that of plasmin and neutrophil lysosomal enzymes. Fibrinogenolysis by this neutrophil membrane protease rendered the fibrinogen non-clottable by thrombin, through cleavage from the N-terminus of the Aα chain and the C-terminus of the Bβ and γ chain. Cleavage of the Aα chain from the N-terminus by the neutrophil membrane protease resulted in a 21 amino acid peptide, Aα1-21, which has previously been reported to be a characteristic of the action of elastase on fibrinogen. This protease was found to degrade fibrinogen during incubation with both non-stimulated neutrophils and neutrophils stimulated with PMA at a concentration which does not release proteases from the azurophil granules. Thus, this study presents an alternative mechanism for the generation of the Aα1-21 peptide which could contribute to the reported plasma levels of this peptide and may also account for the levels of Aα1-21 seen with non-stimulated neutrophils (Weitz *et al.*, 1987).

The results of experiments investigating the mechanism of neutrophil-mediated processing of CRP and fibrinogen indicate that degradation occurs as a consequence of these ligands interacting with the neutrophil membrane. In the case of fibrinogen, and possibly CRP, this interaction was concluded to occur at a position in close proximity to the CD11c receptor of the β2 integrin family. The inability of the association of radiolabelled CRP with neutrophils to be inhibited by unlabelled CRP, in the presence of inhibitors to the membrane protease, would not allow for accurate assessment of the position on the neutrophil surface to which CRP binds. The interaction of both CRP and fibrinogen with the neutrophil resulted in a time dependent egress of the protease from the cell, thus promoting the degradation of these ligands in the extracellular medium. The products generated were found to associate with the neutrophil surface and in the case of fibrinogen degradation products this association was with the receptors of the β2 integrin family and the IgG Fc receptor, CD16. The CRP degradation products did not appear to bind to receptors of the β2 or β3 integrin family or the IgG Fc receptor.

Although maximum degradation of both CRP (Shephard *et al.*, 1989) and fibrinogen by the neutrophil membrane-associated preotease was achieved by stimulating the cells with PMA, these ligands could be degraded in the presence of non-stimulated cells. This was especially so in the case of fibrinogen degradation. The interaction of fbg could activate cellular kinase activity *per se* while kinase activation during the interaction of CRP with neutrophils required stimulation with PMA. The increase in proteolytic activity of the membrane-associated protease following ligand binding and PMA activation involves both H7 and TFP inhibitable kinases in addition to alterations in the microfilament structure of the cytoskeletal network. Protein kinase C does not, however, appear to be involved in the upregulation of this enzyme.

Although the exact structure and sequence of this neutrophil membrane protease was not determined, it appears to be distinct from the only other serine protease (300 kDa) which has been described to be associated with the neutrophil membrane and cytoskeleton (Pontremoli *et al.*, 1986; Pontremoli *et al.*, 1990). This 300 kDa protease is involved in the downregulation of protein kinase C and is completely released into the extracellular medium as a result of PMA activation and protein kinase C stimulation (Melloni *et al.*, 1986). A membrane bound form of proteinase 3 as well as elastase and cathepsin G have recently been discovered (Csernok *et al.*, 1994; Bangalore and Travis, 1994). These proteases within the neutrophil granules have considerable amino acid

sequence homology and all possess elastase-like activity (Rao *et al.*, 1991). They are, however, distinct on the basis of their specific catalytic properties and sensitivity to certain inhibitors. The protease described here may represent another member of this family of elastase-like proteases. The fact that this enzyme exists as a large molecular weight complex may account for it behaving differently from elastase, with respect to cleavage specificity and sensitivity to inhibition by elastase inhibitors.

The generation of non-clottable fibrinogen through the action of a membrane associated protease could well influence the extent of fibrin deposition at sites of inflammation. In addition, the ability of fibrinogen degradation products of less than 30 kDa to associate with receptors important for neutrophil function, could influence neutrophil adhesive and phagocytic processes. When this is considered together with the previously observed generation of bioactive CRP peptides which inhibit neutrophil inflammatory functions by this neutrophil membrane enzyme (Shephard *et al.*, 1989; Shephard *et al.*, 1990; Shephard *et al.*, 1992), it is tenable that activated neutrophils may play an important role generating products which limit the extent of an inflammatory reaction.

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